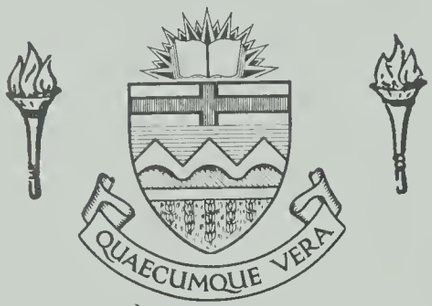


THE UNIVERSITY OF ALBERTA

VELOCITY SEDIMENTATION SEPARATION OF A MURINE ASCITIC LYMPHOMA

BY

© JAN MARGARET DAVIES

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL, 1972

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES
AND RESEARCH

THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES FOR ACCEPTANCE, A THESIS ENTITLED

"VELOCITY SEDIMENTATION SEPARATION OF A MURINE ASCITIC LYMPHOMA"

SUBMITTED BY JAN MARGARET DAVIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (SURGERY)

DATE: September 8, 1972

ABSTRACT

The technique of velocity sedimentation has previously been concerned with separation of cells of immunological importance. Few definitive studies have been reported on the correlation between cell size and chromosome complement, using either normal or neoplastic populations. This study reports the characterisation of a murine ascitic lymphoma as to velocity sedimentation profile, comparison of this distribution with actual cell size distribution, and correlation of cell size with chromosome complement.

A tumour of 5 days *in vivo* culture was sedimented in a non-linear gradient of 3-30% FCS, in a STAPUT apparatus. The resulting profile had a unimodal distribution, mode = 8.7 mm/hour. Tumours obtained after 5 days of *in vivo*, 1-2 days *in vitro* culture were also sedimented. The profile remained unimodal, but the modal value shifted to 6.9 mm/hour. This shift indicated a change in the mode of the population's cell size distribution. After 1-4 weeks in tissue culture, the tumour's sedimentation profile became bimodal (modal values = 6.8 & 17.3 mm/hour), corresponding to micro- and macro-cytic shifts in the cell sizes. A tumour of 7 days *in vivo*, 5 days *in vitro* culture was sedimented and the cells (average diameter = 10μ) of the 100% cell peak were cultured *in vitro* for eight days. Sedimentation of this culture showed a profile with a full velocity range. Thus, the culture was composed of cells ranging from at least 8 - 18μ in diameter.

Actual cell sizes were determined by measurement of cell diameters

under Phase Contrast microscopy. Samples of 5 day *in vivo* tumours and 5 day *in vivo*, 1-2 day *in vitro* tumours were measured and cell size distributions determined. The diameters were of unimodal distribution, ranging from 8 - 18μ, modal value = 12μ, and 8 - 17μ, modal value = 10μ, respectively. These distributions were then converted into theoretical sedimentation velocity distributions (using Stoke's Law) which correlated well with actual sedimentation velocity distributions for the same tumours.

The unsedimented tumour had a chromosome complement of 70% stemline (s), modal chromosome # = 41, 30% double stemline (2s), modal chromosome # = 81-82. Various separated fractions of the 5 day *in vivo* tumour were analysed for 2s cell chromosome complement. The 2s cells were found to have a bimodal distribution at 9.0 & 11.0 mm/hour, corresponding to cell sizes of 12 & 13.5μ. Previous studies had reported cell size to be a function of cell ploidy. These results fail to show evidence of increase in cell size as a result of increase in chromosome complement.

ACKNOWLEDGMENTS

Space does not permit me to acknowledge the many people who have given me help during the past two years, by instructing me in the art of scientific research and by encouraging me when I least thought that science could be 'fun'. Those mentioned below helped me immeasurably and I therefore thank

Olin G. Thurston, M.D., F.R.C.S.(C.), F.A.C.S., for his benevolent and understanding supervision,

Norbert Kraft, Ph.D., who first taught me how to 'run a STAPUT', and without whom this research would not have been,

Maureen Huggins, for excellent technical instruction and assistance,

Sylvia Roberts, M.C.S.P., for deciphering my handwriting, and agreeing with my use of commas,

Harvey Orton, for the excellent illustrations,

Amy Fuller and Janet Larsen, for the typing of the tables and figures, and text, respectively.

This research was supported by a grant from the National Cancer Institute of Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The heterogeneity of neoplastic cells is usually considered to be a definable feature of these cells. Cancer cells are polymorphic: the cytoplasmic/nuclear ratio is lower than that of normal cells and resembles more the ratio of the undifferentiated forms of the cells; the nuclei are bizarre, multi-lobulated and often possessing several nucleoli; the karyotypes exhibit marked diversity, often being aneuploid. Microscopic examination of sections of a solid tumour [Fig. 1] or smears of an ascitic form [Fig. 2] demonstrate apparent difference in diameters of the cells (as determinable by the resolution of the microscope-aided human eye). It therefore seems logical to consider the possibility of separating a tumour into fractions of cells, on the basis of difference in cell size. Because neoplasms are composed of "malignant cells which are served by a non-malignant stroma made up of connective tissue, blood vessels, nerves and lymphatics" [Cowdrey, 1955], then any separation procedure must necessarily separate cells from the malignant and non-malignant portions. Thus, a pure separation of malignant cells will permit more accurate examination of the actual tumour.

In biological systems, differences in cell size are usually equated with differences in cell function. A currently popular theory suggests that, within a (solid) tumour, certain cells are responsible for certain functions [Delarue, 1972]. The different functions of cells within a tumour may be defined as

1) tumour maintenance

2) metastatic ability (tumourigenicity)

3) anti-immunogenicity

4) host death ? - phagocytosis

Thus, if this hypothesis is correct, there must be similar categories of cells, according to their abilities to carry out these functions. And because the premise of cell size defining cell function has previously been mentioned above, logic now demands that if a tumour be separable into cells of different sizes, then cells of different functions may be so separated.

A technique which employs the difference in cell sizes to achieve separation is Velocity Sedimentation. The theory of this technique is based on Stoke's Law:

$$s = \frac{2gr^2(d_c - d_f)}{9\eta}$$

If cells are subjected to a unit gravity force (g) in a fluid medium of lower density (d_f) than the least dense cell (d_c), then the cells will settle, toward the bottom of the sedimentation vessel. Settling will occur at a rate (s) determined by the force of gravity (g) and proportional to the square of the radius (r) of the cell. Viscosity (η) will also influence the rate of fall by exerting a drag force on the cell and therefore decreasing the velocity. Because of the lower density of the fluid medium, the cells will continue to settle until they reach the bottom of the vessel. However, before this point in time is reached, if serial fractions are removed from the vessel, (sampling vertically), then a distribution of the experimental

population may be demonstrated, according to sedimentation velocity difference and therefore to size difference (radius).

Such a technique could, as mentioned above, be used to acquire pure samples of tumour cells for critical analyses. Or, this technique could be employed in cell hybridisation studies, where separation of the hybrid cells from the parent populations is required. However, this separation would be made easier if one of the parent populations was well characterised as to sedimentation profile. This latter application of the technique of Velocity Sedimentation is the purpose of this study. The work described here is the first part of a larger project, the aim of which is to study the growth of a tumour of mouse/human hybrid cells *in vivo* and *in vitro*.

REVIEW OF THE LITERATURE

Historical Review of Cell Separation Methods

The concept that suspensions of cells could be separated by simple sedimentation into fractions containing cells of differing properties has been known for several centuries. The ancient Greeks were aware of the fact that blood (a cell suspension), if left undisturbed for some thirty minutes, would become a thick red band overlain by a cloudy liquid. This rapid settling of the red blood cells, in contrast to the other formed elements of the blood, leading to separation of the red blood cells, was known to Hippocrates (in terms of phenomenon, rather than as an actual mechanism). Red cell sedimentation also formed the "basis for the humoral theory of diseases ... until the advent of Virchow's cellular pathology" (Hirschboeck, 1947). Later, in the 18th and 19th centuries, physicians realised the importance of the differences in rate of this differential cell separation in health and disease [Ponder, 1947].

It was not until 1918, however, that the mechanics of blood cell separation were explained. Fåhreus showed that the rate of red blood cell separation was proportional to the "degree of rouleaux aggregation among the erythrocytes" (Hirschboeck, 1947). This discovery agreed with the terms of Stoke's Law: in a cell suspension in which the radius of the cell is the only variable, increase in degree of rouleaux aggregation would increase the radius and therefore the rate of sedimentation.

As a result of Fåhreus' discovery, many workers attempted to modify red cell sedimentation in order to achieve separation of all the different blood cell types. Addition of fibrinogen (inherently part of the rouleaux formation process) accelerated red cell sedimentation. This was later abandoned in favour of dextran, the use of which avoided "formation of lumps of fibrin with adherent white cells or platelets" [Alexander & Spriggs, 1960].

After the use of these sedimentation-enhancing agents became established, lymphocyte-rich solutions of reasonable purity were available, for biochemical and morphological tests. These tests had previously been attempted on solutions of lymphocytes, for example, which might be contaminated with six red blood cells to every white blood cell [Cutts, 1970]. Thus, Fåhreus' work not only explained the phenomenon of red cell sedimentation, but also made possible differential cell separation.

In order to improve the separation of different cell types, many other techniques were devised, a short review of which is given by Pretlow *et al.* [1971]. Phytohaemagglutinin (PHA), extracted from the red bean *Phaseolus vulgaris*, was added to blood to agglutinate the red cells. These large clumps were sedimented under gentle centrifugation, yielding a supernatant fluid rich in nucleated cells [Li & Osgood, 1949].

One of the most fascinating methods of cell separation involved use of magnetism. The experiment was originally undertaken in an attempt to separate the red cells by use of the "paramagnetic properties

of haemoglobin" [Levine, 1956]. Haemoglobin possesses a paramagnetic value of 4.90 Bohr magnetons, which Levine thought would be strong enough to attract the red blood cells into a magnetic field. This experiment failed, but Levine achieved magnetic separation of the blood cells. Starch-coated iron particles were added to the blood and were promptly phagocytised by white blood cells. These magnetic phagocytes stuck to a paraffin-coated petri dish mounted over an electromagnet. The blood proteins and red cells were washed out, leaving a clean separation of phagocytic white cells. This experiment had previously been tried by Kuper *et al.* [1961]. Samples of blood from patients with tumours were subjected to red cell lysis with saponin and removal of the polymorphonuclear leukocytes with a magnet, after the white cells had ingested iron particles. The residual cells were fixed, after filtration onto a Millipore membrane, then stained with Haematoxylin and Eosin, cleared, and mounted in synthetic resin. This technique facilitated the estimation of the number of tumour cells present in the peripheral blood and the blood draining the tumour sites.

Various forms of filtration were used to remove unwanted cells from suspensions. Cudkowicz *et al.* [1964] passed bone marrow through glass wool columns, collecting an eluent of only lymphocytes, cells "capable of continuous proliferation" and considered to be pluripotential cells. Filtration, through Garvin's glass bead columns, of dextran sedimented leukocyte syspensions, resulted in separation of polymorphonuclear leukocytes and monocytes, from each other and from the other blood cells [Rabinowitz, 1964]. Abdou & Richter [1969] used Ag-sensitised glass bead columns to isolate Ag-reactive cells in normal rabbit

bone marrow from a suspension of marrow cells. Similarly, Wigzell and Andersson [1969] used glass and plastic bead columns coated with antigenic protein molecules as "immunological filters for a cell population containing immune cells of relevant specificity".

Exploitation of the "differential adhesive property of lymphocytes and monocytes" was carried out by Bennett & Cohn [1966] to separate monocytes (adherent to the walls of Leighton tubes) from lymphocytes (washed out with tissue culture medium). A similar technique was used by Bloom & Bennett [1966] to study peritoneal exudate macrophages and by Mosier [1967] to study Ab formation to sheep red cells by normal spleen cells.

Electronic separation of cells was developed by Fulwyler [1965] who separated mixtures of mouse and human red cells. A modified Coulter counter was used, with the cell volume measured in the Coulter aperture. The cells were then isolated in drops of diluent. Each drop containing a cell was given a charge, depending on whether or not the cell size exceeded a certain volume. The "charged droplet then entered an electrostatic field and was deflected into a collection vessel". The cells were therefore separated into two size groups, large and small. This technique was later used by VanDilla *et al.* [1967] who demonstrated the bimodal distribution of normal white blood cells of the human male.

Probably the most widely used method of cell separation is centrifugation which was developed as an attempt to accelerate gravitational-force separation. Differential gradient centrifugation separates cells on the basis of density alone. The cells are layered over a density gradient and then centrifuged until they reach their isopycnic

density layer. Further centrifugation does not alter their position in the density gradient. An excellent review of the work prior to 1959 is given by DeDuve *et al.*[1959]. Yet despite the method's basic simplicity (as it was performed then), as Goodman [1960] noted, centrifugation did not meet the "requirements of purity, high yield, and cell viability". By 1963, however, results from density gradient centrifugation had improved, as many of the theoretical considerations had been resolved. For example, DeDuve reported on the sedimentation coefficient, a "complex function of size, shape and density of the particles and density and viscosity of the supporting medium" [DeDuve, 1963]. In 1964, Leif and Vinograd reported the buoyant cell distribution of human red blood cells. This paper was of major importance, being perhaps the catalyst of the development of centrifugal cell separation as it is known today. First, the cells were separated in Bovine Serum Albumin, thus getting away from the osmotic effect of high sucrose concentrations. Second, Leif and Vinograd determined that the erythrocytes behaved as "perfect osmometers", thus establishing the red cell as an osmotic marker. In 1964, Hilal and co-workers reported achievement of high-resolution in their system, allowing separation of cells with density differences as small as 0.22 gm/cm^3. They also reported use of dextran as a gradient material, citing it as being physiologically inert and a good agent for cell dispersal [Hilal *et al.*, 1964]. Much excellent work in this field has been done by Shortman, one of whose papers [Legge & Shortman, 1968], reported on the effect of pH on the size, density and shape of red blood cells and thymic lymphocytes. This paper discussed the difference between human and rat erythrocytes, both of which varied

in volume with pH, but to a different degree, and thymic lymphocytes, which did not vary with pH. Legge and Shortman were therefore able to recommend the need for "control of pH as well as tonicity when using red blood cells to calculate Coulter Counter apertures or when performing size range or density distribution analyses of the erythrocyte population". Since then, differential gradient centrifugation has become well established, allowing, for example, enrichment of fractions for Colony Forming Units, partial elimination of Graft versus Host activity, and subsequent "protection of allogeneic irradiated recipients without acute or delayed secondary disease" [Dicke *et al.*, 1968].

A second method of centrifugal cell separation is rate-zonal sedimentation. Cells are centrifuged to separation which is determined by differences in both diameters and density. Pretlow and co-workers found separation to be possible even when these differences were as little as 1μ or 0.010 gm/ml [Pretlow *et al.*, 1969 abstr.], a considerable improvement over the limits of high-resolution achieved by Hilal and workers in 1964. This method was developed because of the theory that diameter was more important than density as a means of separating cells since different cell types differed more with respect to diameter than density [Pretlow & Boone, 1969]. Since then, Pretlow and co-workers have achieved successful separation of various mammalian cell types, including mast cells in successive stages of differentiation [Pretlow & Cassady, 1970].

Thus centrifugation in its various modifications has been shown to be a most successful method of cell separation. Unfortunately, there are so many methods of, and variations on, simple centrifugation, and

so much work has been done in this field, that to discuss them all would surpass the limits of this discussion of cell separation.

Development of Velocity Sedimentation Method of Cell Separation

Another method of cell separation, and the major topic of this thesis, is velocity sedimentation. This is a relatively new technique, having its origins in 1959, when a biophysicist, H.C. Mel, reported a new method of continuous free boundary electrophoresis. The method allowed "complete, continuous separations at high flow rates in a compact apparatus" [Mel, 1959]. The significance of this report remained obscure to all, perhaps, but those interested in electrophoresis, until 1960, when Mel reported again on this method of electrophoresis. This method now allowed the "study of interactions in free solutions, including weak interactions, by rapid mixing and unmixing accompanied by low-stress separations of reactants and products" [Mel, 1960]. This was interesting, but not altogether pertinent to those interested in cell separation, and the report would have remained so, except for one clause. The method was also considered "applicable to small molecules, large molecules and cells". This then was the beginning of the separation method known today as velocity sedimentation.

Mel's progress in the field continued at a rapid rate. In the first of two papers published in 1963, Mel described the sedimentation, under 1g only, occurring "vertically at right angles to the flow" of the cell suspending medium [Mel, 1963a]. The next paper considered the mathametics (Stoke's Law) of cell sedimentation in good detail. He showed that, despite the fact that the greater density of mature red

cells clearly favoured their more rapid settling, the size factor was "much more important than the density factor". This therefore caused the more rapid sedimentation of the white blood corpuscles than of the red blood corpuscles. He also reported the separation of the non-nucleated and nucleated cells of the bone marrow. This separation, "subject to 1g only", was dependent "strongly on cell size" [Mel, 1963b].

In 1964, Mel published three papers on the "stable-flow free boundary system", nicknamed STAFLO. The first paper considered the apparatus and its hydrodynamic feedback principles. The STAFLO consisted of a lucite flow cell, 30 cm long, 3 cm high, 0.7 cm wide, with twelve inlet-outlet combinations of 0.25 x 0.7 cm cross-sectional area. Each chamber had a volume of 5.25 ml, one-twelfth of the total 63 ml chamber volume. The suspensory solution flowed continuously with rate controlled on a feedback principle. This gave a "free solution inherently stabilised against convection". This was a most important factor in the separation process, the *sine qua non* of (which was) stability of flow" [Mel, 1964a].

The stability and laminar flow of the density gradient were the topics of the next report. The stability resulted, in part, from the fact that a density gradient under only gravitational force was subject solely to diffusion effects. If the gradient was hydrostatically stable, then diffusion, and therefore convection (instability), would be minimal. Gradient stability was also due to the laminar flow, a product of the feedback flow control system mentioned above [Mel, 1964b].

However, possibly the most important part of the paper was Mel's description of "clump sedimentation (or familiarly - bombing)". This phenomenon was also known as "sedimenting droplets", "streaming droplet

sedimentation", "diffusion-induced micro-convection" [Mel, 1964b], or simply "streaming". Streaming occurred when clumps of cells broke away from the main band of cells and rapidly sedimented downwards. These clumps were "usually loose enough to leave a trail of cells behind, clearly marking their trajectories" [Mel, 1964b]. The phenomenon was well described by Miller and Phillips [1969] as an "upside-down grass lawn". Mel thought that streaming was due to the density of the sample and the solute suddenly being much greater than the density of the gradient below. This would appear logical if two controlling factors were considered. These were the number of cells/ml of loading volume (streaming limit) and the "state of dispersal or aggregation of the cells in the sample suspension" [Mel, 1964b]. The streaming limit varied for each cell type. Mel found it to be 4.3×10^7 cells/ml for yeast, whereas Miller and Phillips [1969] found 3.3×10^6 cells/ml to be the limit for mouse spleen cells. There appeared to be a general rule that the limit was "inversely proportional to the average volume of the cells being sedimented" [Miller & Phillips, 1969]. Britten and Roberts [1960] also determined that the amount of sample which could be handled increased "with the square of the width of the sample layer, since it (was) the gradient in density and not the maximum density of the sample which (determined) stability". As for the state of dispersal of the sample cells, in a cell solution of random distribution, some cells would be much closer to neighbours than others. Decreasing the sample volume would lead to an increase in the occurrence of this proximity. A combination of cell load greater than the streaming limit and a small loading volume could produce "bombing" [Mel, 1964b].

In the third paper, Mel [1964c] considered various principles of sedimentation. He discussed the "velocity dependent Stoke's frictional force" and its implications in the case of non-spherical shapes. Mel was of the opinion that a correction factor should be included in velocity calculations. This factor was to account for variations in cell shape or size during sedimentation in different gradients.

By 1965, Mel had enlarged the purpose of the STAFLO to encompass "unambiguous, reproducible physical classification of the cellular constituents in the heterogeneous mixture ... and preparative separation of large numbers of cells of different types, for direct investigation of their characteristic biological ... properties" [Mel *et al.*, 1965]. He and his colleagues had also fractionated a single suspension of normal rat bone marrow, resulting in "large relative enrichments ... for certain cell types". Sedimentary separation was now well established. Then, in 1967, the first modification of Mel's system appeared. Peterson and Evans [1967] described a conical-ended chamber, of 20 cm diameter. The bottom inlet port connected, out of line, to a cell application chamber, and on line, to a three-compartment gradient maker. The top outlet port connected to a fraction collector and fractions were collected by means of upward displacement, in order of increasing sedimentation rate. Using a non-linear gradient of sucrose, at 4°C, they separated guinea pig bone marrow into erythroid and myeloid components. They also separated "blasts, neutrophil myelocytes, and mature neutrophils", with about 90% recovery of applied cells.

The system of Peterson and Evans differed from that of Mel in that the gradient fluid flow occurred in the same direction as the

force of gravity, whereas, in the latter, fluid flow was at right angles to gravitational force. This fact perhaps helped to account for the better separation achieved by Peterson and Evans than by Mel.

Peterson and Evans also discussed "streaming", agreeing with Mel on the description and cause of the phenomenon. The former, however, placed sole emphasis on the cell concentration (and distribution) of the loading sample, as the critical factor. Yet Peterson and Evans suggested that a shaped gradient, with a sheer step in concentration from 0.3 to 1% and then a linear rise in concentration, could be used as a method of minimizing "streaming". Presumably, this concentration shift reflected a density shift, thus reducing the chances of the density of the sample and solute suddenly becoming much greater than that of the gradient below.

The first application of velocity sedimentation to immunological research was achieved by Mage, Peterson and Evans in 1968. They used a sedimentation chamber of 16" diameter, with a 4800 ml sucrose gradient. Nucleated spleen cells (1.4×10^8 total cells) were sedimented for 100 minutes at 4°C. Upward displacement yielded 50 ml samples, with enrichment for cells forming haemolytic antibody plaques. This enrichment of plaque versus nonplaque-forming cells occurred at up to 18 times the initial concentration.

The next report on velocity sedimentation was one of the most important, for it considered many relevant factors involved in cell separation. Miller and Phillips [1969] formally advanced the concept that cell separation, according to differences in cell size, could be both preparative and analytical. Cells from separated fractions could be subjected to many tests, biochemical and morphological. Also, the

separated fractions could be given a value for rate of settling ("s value"). A (whole) population could therefore be defined as to its distribution ("sedimentation profile") when subjected to velocity sedimentation.

They also discussed the function of the density gradient through which the cells were separated. Of a density lower than the least dense cell, the gradient did not function to separate the cells on an isopycnic basis. Rather, the density gradient functioned "to prevent mixing of adjacent layers ... and ... convection and mixing of adjacent layers during loading and unloading of the sedimentation chamber". This gradient could have one of two forms. The buffered step gradient had an initial zone of 3% fluid concentration in which the cells were suspended, a jump to 5%, a steep rise to 15%, and then a slow rise to 30%. The sheer step gradient had an initial zone of 5% fluid concentration, in which the cells were suspended, a jump to 15%, and then a linear rise to 30%. Miller and Phillips found that the "streaming limit" ["critical cell concentration" (of loading sample) above which "streaming" occurred] was four times higher for a buffered step gradient than for a sheer step gradient. This discovery was vital in the planning of experiments, for use of the right gradient allowed a higher cell concentration in the loading sample. Such manipulation therefore decreased the importance of the choice between "diminished resolution" (as a result of streaming) or extension of "sedimentation time that accompanied use of a larger volume of sample" [Peterson & Evans, 1967].

As their gradient material, Miller and Phillips used Fetal Calf Serum (FCS) in Phosphate Buffered Saline (PBS), in contrast to Peterson and Evans [1967] and Mage *et al* [1968] use of sucrose. Several

important physico-chemical values for FCS were defined. The protein concentration for a solution of 30% FCS was found to be only 1-2%. Over a range of 0-30% FCS, the density shifted from 1.004 to 1.009 gm/cc. Comparison of this density range to the typical density of a nucleated cell (1.060 gm/cc) ensured that the density gradient did not interfere in the sedimentation of the cells. Miller and Phillips also defined the viscosity of FCS at 4°C to be similar to that of H_2O (1.56×10^{-2} poise) at the same temperature. This value has, however, been disputed, with actual measurements showing 1.72×10^{-2} poise to be a more appropriate value [Kraft, 1972].

Miller and Phillips introduced a slight modification to Peterson and Evans' system. In the new system, the sedimentation chamber became a simple cylinder, tapering to a cone (at an angle of 30° to the horizontal). All input and output manipulations were carried on through the bottom cone outlet. Miller and Phillips also named this new chamber as a STAPUT, deriving the name from Mel's STAFLO, from which their system had evolved. (The name STAPUT has since come to represent not only the sedimentation chamber, but also the method of cell separation.)

Mouse L-cells were loaded into the STAPUT, under a layer of Phosphate Buffered Saline (PBS), which theoretically functioned to prevent "disturbance of the cell band by erratic movements of the rising fluid meniscus" during filling of the STAPUT. The murine cells, of radius 7.5μ, were found to have an "s" value of 12 mm/hour.

Shortly after the publication of the Miller and Phillips paper, Worton and co-workers reported the "physical separation of hemopoietic stem cells for cells forming colonies in culture" [Worton *et al.*, 1969].

Using a STAPUT of 11.3 cm diameter and 7 cm cylindrical height, they loaded 4 x 10^6 bone marrow cells in 20 ml medium under a 30 ml medium overlay. This was followed by a 450 ml linear gradient of 15-30% FCS in medium. This gradient represented neither a sheer- nor a buffered-step gradient. Yet, after collection of 26-28 fractions of 15 ml each, Worton reported some separation of cells forming colony units in culture (CFU-C) from cells forming colony units in the spleen (CFU-S). This was confirmed by density centrifugation separation of the cells, with similar CFU-C, CFU-S resolution.

From this point on, velocity sedimentation was applied almost exclusively to the field of immunology. In the first of two 1970 papers, Miller and Phillips reported a "sedimentation analysis of the cells in mice required to initiate an *in vivo* immune response to sheep erythrocytes" [Miller & Phillips, 1970a]. They gave "s" values of 4.7 mm/hour for 19s (svedberg) Ab-producing cells, 4.4 mm/hour for 7s (svedberg) Ab-producing cells, 2.0 mm/hour for erythrocytes, and 4.3 and 5.9 mm/hour for two granulocyte fractions. The immunocompetent cells of mouse spleen, bone marrow, and thymus and the rosette-forming cells of the bone marrow and spleen all sedimented at a rate of 3 mm/hour. Miller and Phillips postulated that the 3 mm/hour cells might be the "direct progenitors of Ab-producing cells".

In a study of "physiological and radiobiological properties of immunologically reactive mouse spleen cells" Osoba [1970] used two STAPUTS, of 17 and 21.5 cm diameter, loading 5 x 10^{6-7} cells/ml in 3% FCS. Osoba was able to partially separate the three classes of "immunologically reactive cells required for the production of Plaque Forming

Cells (PFC) in culture". The rosette-forming cells had a peak velocity of 3.2 mm/hour. Their immunological activity (response to foreign red blood corpuscles) was inhibited by low doses of irradiation. The non-rosette-forming cells had a peak velocity of 3.6 mm/hour. These cells facilitated production of haemolysin-forming cells by small numbers of normal spleen cells. Large doses of radiation had no effect on this function. The haemolysin forming cells had a peak velocity of 4 mm/hour. These cells, which Osoba thought were possibly direct descendents of the rosette-forming cells, did not form rosettes. Their function (haemolysin formation) was, however, inhibited by low doses of irradiation, as was the function of the rosette-forming cells.

Miller and Phillips, in their second 1970 paper, did not discuss results but considered general principles of analysis and purification by velocity sedimentation of Ab-producing cells. They stated that the rate of settling of cells was "directly proportional to $(\text{volume})^{2/3}$". But because a cell's volume was altered by such factors as the osmotic pressure of the fluid, the sedimentation rate would be similarly altered. Therefore, well-defined conditions were essential for reproducible sedimentations. Once conditions were established, velocity sedimentation could separate a population into fractions "significantly enriched for Ab-producing cells" [Phillips & Miller, 1970b]. The resulting sedimentation profile could be used in the study of the cells' proliferative states and different functions.

With the establishment of velocity sedimentation as a well-defined and reproducible technique, it became possible to use size separation before or after analysis of, for example, cell density.

Haskill and Moore, in 1970, used such a two-dimensional separation in a comparison of bone marrow stem cells from embryos and adults. Density gradient centrifugation was followed by velocity sedimentation of cells of density 1.0565 - 1.0585, in a gradient of 4-30% FCS, at 4°C for 2½ (embryonic cells) or 4 hours (adult cells). They felt that use of both techniques was mandatory with a tissue such as bone marrow, which represented a "complex mixture of cells varying in size and density". [Haskill & Moore, 1970]. Use of both size and density separation enabled them to conclude that age brought about a differentiation process in embryonic stem cells which was reflected in changes in size and density. These changes were so profound that the "stem cells of early embryonic type (were) absent from adult resting marrow".

The latest paper concerned with velocity sedimentation reported the "synchronisation of mouse L-cells" [Macdonald & Miller, 1970]. On the basis that cell volume increased "monotonically" as the cell progressed through the cell cycle, they sedimented the tumour for 3½-4 hours through a linear gradient of 15-30% FCS in PBS at 4°C, in a STAPUT of 13.8 cm diameter. Fractions were analysed for cell numbers and volume distribution, and subjected to autoradiography. The cells (unsedimented) were also subjected to density centrifugation, and the different fractions analysed for density, cell numbers, cell volume and tritium uptake. Sedimentation analysis showed enrichment of fractions for S and M phase cells. Density analysis showed homogeneous distribution, with an average of 1.051 gm/cc, and a "roughly constant" tritium uptake. Therefore, by separating the cells into the different stages of the cell cycle, synchronization of cells for phases of the cell cycle could theoret-

ically be obtained.

Separation Studies of Neoplastic Cells

Of the papers discussed above, very few were studies on neoplastic cells. Kuper *et al.* [1961] attempted merely to separate tumour cells from the peripheral blood by magnetism, in order to estimate the number of neoplastic cells in the blood. Miller and Phillips [1969] used mouse L-cells, but restricted their reporting of results to cells of radius 7.5μ. Similarly, Macdonald and Miller [1970] separated a population of mouse L-cells, not in an attempt to correlate any specific tumour property with cells of a separated fraction, but because the tumour constituted a general population, easily obtainable and submitting well to study.

There have been a few other studies on separation of tumour cells, but all have involved the use of centrifugation. Pool and Dunlop [1934] were perhaps the first to attempt to separate tumour cells from blood cells. Their degree of separation was really no more than increasing the ratio of tumour to blood cells, as they wished to determine "whether cancer cells (might) be identified in the circulating blood of living subjects". Whereas Pool and Dunlop [1934] wished to develop a system for rapid diagnosis leading to more accurate differential diagnosis of malignancy, Engell [1955] hoped to determine the degree to which "surgical manipulation in the removal of the tumour (might) influence the haematogenous spread of cancer cells". However, the methods used were similar. After centrifugation of hemolysed blood, the pellet of cells was fixed, embedded, cut in sections, mounted, and stained for histological examination. Alexander and Spriggs [1960] attempted to

further improve diagnosis of blood-borne neoplastic cells. They used dextran-sedimentation to produce white cell concentrates which were then treated as above. Results were similar in all three studies: diagnosis was facilitated.

Fawcett and co-workers reported on the first density gradient centrifugation studies. Centrifugation of bloody, pleural and peritoneal fluids yielded malignant cells concentrated at a density of 1.05-1.06 gm/cc. These cells were stained as smears, but Fawcett *et al.* recommended separation of cells for "chemical and physiological investigation" [Fawcett *et al.*, 1950]. Allfrey reported use of the same method to separate Ehrlich ascites cells from other cells of a peritoneal exudate [Allfrey, 1959]. There have been several recent studies using gradient centrifugation. Pretlow and co-workers have reported three times on the separation of artificial mixtures of normal and neoplastic cells. Pretlow and Boone [1968a] described separation of equal numbers of HeLa cells and rabbit thymocytes with 98.5% and 89.0% purity, respectively, followed by successful regrowth of the cells. Pretlow and Boone [1968b] reported separation of Ehrlich ascites tumour cells and HeLa cells and of rabbit thymocytes and Ehrlich ascites tumour cells, the latter separation with 98-100% purity. Finally, Pretlow, Boone and Riley [1969] obtained "cells of trypsin disaggregated, transplantable mouse neoplasms ... with greater than 90% purity".

Boone *et al.* [1968], using isopycnic-zonal centrifugation (separation dependent on cell density only) separated mixtures of rabbit thymocytes and HeLa cells with 99 and 98% purity, respectively, of thymocytes and suspension-cultured, human acute leukemia cells with 93 and 91% purity, respectively, and of HeLa cells and horse leukocytes with 92%

purity for the former cells.

The latest paper concerns the separation of human leukemic blasts from other blood cell types by density gradient centrifugation. Both fresh and frozen cells were used and recovered with 58% and 45% purity, respectively, from the other cells [Abeloff *et al.*, 1970].

Thus, review of the literature shows that very few definitive experiments have been attempted on velocity sedimentation separation of a population of neoplastic cells, either for purely separatory purposes or for further study of separated fractions with respect to tumor characteristics. The author, therefore, decided to examine the sedimentation charactertistics of the cells of the murine lymphoma, L5178Y, clone FFD.

Preliminary experiments with lymphoma cells from *in vitro* cultures were undertaken to see if the cells could be subjected to velocity sedimentation at 4°C and still remain viable. The quality of cell growth was determined on reculturing of different fractions, by cell numbers and the Trypan blue dye exclusion test. Success of these experiments therefore allowed studies of three-fold purpose:

(1) determination of the sedimentation profile of the *in vivo* cell population and of various *in vitro* cultures

(2) phase contrast microscopy measurement of diameters of cells from *in vivo* and *in vitro* cultures

(a) comparison of these measured diameters to those determined by calculation (Stoke's Law) from the sedimentation velocities

(b) calculation of theoretical sedimentation velocities from measured diameters, and comparison of these

rates to those measured in the experiments

(3) determination of the karyotype of the separated fractions.

A diagramatic presentation of the experimental protocol is shown in Figure 3.

MATERIALS AND METHODS

Murine lymphoma cells, rather than human fibroblasts, were used for the sedimentation experiments because

(1) the lymphoma population possesses great variance in its cell size range, thus suggesting a widely distributed sedimentation profile

(2) the lymphoma's bimodal chromosome complement provides a genetic marker for cell fraction analysis

(3) the lymphoma grows very rapidly and therefore the large number of cells required for each sedimentation are easily obtainable.

L5178Y (FFD) Murine Lymphoma Cell Line

The L5178Y lymphoma cell line was originally obtained from Dr. G.A. Fischer, Brown University, Providence, Rhode Island, through the courtesy of Dr. A.R.P. Paterson (McEachern Laboratory for Cancer Research, University of Alberta). The clone FFD was derived by Chong Kim (McEachern Laboratory).

The lymphoma cell line was maintained in female and male BDF_1 mice (Microbiological Associates, Inc., Bethesda, Maryland) by weekly intraperitoneal passaging of 2×10^7 cells obtained from ascitic fluid.

In Vivo Retrieval

On the fifth day after innoculation the mice were killed by cervical fracture and the ascites removed with needle aspiration, according

to the following procedure. The abdomen was swabbed with 70% methanol. 5 ml of warm (37°C) Fischer's medium for Leukaemic cells of mice were injected into the peritoneal cavity and then the entire ascitic contents aspirated. This was repeated twice, using fresh medium each time. Total yield of ascitic fluid was about 5 ml. The cells were at first retrieved by simple needle aspiration. However, results of preliminary experiments showed that the above procedure was necessary.

Preparation of Cells for Tissue Culture and Sedimentation

The aspirated ascites were centrifuged at 4000g for five minutes. The supernatant was poured off and the cells resuspended in 20 ml of warm medium and re-spun, to remove contaminating red blood cells. If the tumour sample was heavily bloody, the cells were instead resuspended in 5 ml of medium and 15 ml of Hank's Balanced Salt Solution, and incubated at 4°C for five minutes. Clumps of red blood cells were removed by drawing up small volumes of tumour sample into a Pasteur Pipette. Standing of the pipette caused the clumps to settle to the narrow tip. The clumps could then be ejected with very little loss of tumour sample. The cells were finally re-centrifuged and resuspended in 20 ml of warm medium for counting, before culture or sedimentation.

In Vitro Maintenance of Cell Line

The cells were grown in suspension in Fischer's Medium for Leukaemic Cells of mice, supplemented with 15% horse serum, and 100 units and 100 mcg of penicillin and streptomycin, per 100 ml of medium, respectively. If large numbers of cells were required, a 500 ml spinner culture flask was used. The medium was at first changed three times weekly, Monday, Wednesday and Friday. However, results of preliminary experi-

ments showed that the cultures required a change of medium every day to keep the population at maximum growth rate.

Solutions for STAPUT

Gradient Solutions

Four solutions, of 30%, 15%, 5% and 3% Fetal Calf Serum in Phosphate Buffered Saline, formed the gradient through which the cell separation was effected. 600 ml each of the 30% and 15% solutions and 55 ml and 60 ml of the 5% and 3% solutions, respectively, were required for the 1315 ml gradient.

The Fetal Calf Serum (Grand Island Biological Company, Berkeley, California), (stored at 0°C), after thawing, was filtered through a Millipore Filter(R) (0.3μ, 47 mm diameter, plain white) and re-frozen in aliquots of 275 ml (amount needed for one experiment). The FCS was not re-thawed until just before an experiment. The serum was then cooled to 4°C and mixed with the requisite amounts of PBS.

The Phosphate Buffered Saline was prepared according to Dulbecco and Vogt [1954]. The three solutions (A, B, C) were prepared and autoclaved separately, and when cooled, were mixed [Table 1].

Gradient Support Solution

600 ml of dense liquid were used to raise the FCS/PBS gradient out of the cone and into the vertical portion of the STAPUT. This support solution was prepared by adding 15 gm of high molecular weight powdered dextran (average molecular weight = 117,000 gm/mole) (Sigma Chemical Company, St. Louis, Missouri) to 600 ml of Fischer's Medium for Leukaemic Cells of Mice (as for tissue culture). This solution, when

cooled to 4°C, had a density of 1.020 gm/cm^3 [as measured with a densitometer (Fisher Scientific, Edmonton), which permitted a three-decimal figure to be read].

Cell Separation

Materials

Cell separation was carried out in a STAPUT or velocity sedimentation system, at 4°C. The velocity sedimentation system had three basic components. They were the "Gradient Generating Components", the "STAPUT", and the "Collection Components" [Fig. 4].

The "Gradient Generating Components" consisted of three bottles, numbered 1, 2, 3 from left to right, and containing the gradient solutions, 30%, 15% and 5% FCS in PBS, respectively. The bottles were connected in line, and to the "STAPUT" and "Collection Components", by short lengths of silastic tubing, and shut off with surgical clamps. Two magnetic stirrer boxes were required, under Bottles #2 and #3. Bottle #1 was simply supported at the same height. Bottle #3 was tightly stoppered during gradient formation.

The STAPUT was that part of the system where the sedimentation took place. The STAPUT used in these experiments was of 230 mm diameter, 27 mm vertical depth, and 600 ml cone volume. The vessel was siliconised with a 10% solution of Dri-film (Pierce Chemical Company, Rockford, Illinois) in CCl_4, followed by repeated washing in dH_2O, before each experiment. A flow control valve (Fischer and Porter Ltd., Toronto, Ontario) was positioned on line immediately after the Y-piece, and close to the STAPUT for ease of control.

The "Collection Components" were provided by the Y-piece to which a Pasteur Pipette was attached, via a short length of tubing. The tubing was shut off by a surgical clamp until collection.

Methods

The three bottles were loaded with fluid and then clamped off, Bottle #3 remaining unstoppered.

Bottle #3 was then allowed to empty so that the tubing to the STAPUT was filled to the base of the STAPUT cone. There was still some fluid in Bottle #3, enough to cover the magnetic stirrer bar, and at least 0.5 inch of fluid above the exit port level.

The flow control valve was closed off and Bottle #3 corked tightly. The cell suspension (3% FCS in PBS) was poured into the STAPUT, the fluid level almost covering the baffle. The stirrers were switched on, the clamps released in order #1, #2, and flow control valve, and timing started.

The gradient was formed slowly at first, at a rate of flow of 5 ml/min. Once the cell band had completely cleared the baffle, the rate of flow was increased to 30 ml/min. When almost all the gradient fluid had flowed into the STAPUT, Bottle #1 was clamped off and the dextran uplift fluid poured into Bottle #2, and allowed to run through Bottle #3 and into the STAPUT.

When all the fluid was in, the system sat for n minutes. The tubing was then clamped off, just before the Y-piece. The collection outlet was unclamped and serial fractions of 30 ml each were taken at a rate of 1 fraction/minute, into sterile, graduated, tissue culture tubes

(Bellco Glass Company, Maryland).

Times were taken when the fluid reached the top of the cone, the dextran entered the base of the STAPUT, all fluid was in (plus any waiting time), the dextran was emptied, the fluid reached the base of the vertical section of the STAPUT, and sampling was finished.

Cell Counts

Haemacytometer

The sample was diluted with an equal volume of 0.04% Trypan blue dye in Hank's Balanced Salt Solution, for determination of cell numbers and viability. After five minutes at 20°C, the sample was counted, at a magnification of 250X, using a Neubauer haemacytometer chamber. The number of cells taking up the dye (non-viable) was never greater than 5%, when the tumour was freshly removed from the mouse, except in the case of Experiment 29.

Coulter Counter

A model F Coulter Counter (Coulter Company, Hialeah, Florida), with settings at: Gates 10 → ∞, ^{1}/Amplification = 2, ^{1}/Aperture = ½, was used to determine the number of cells in each separated fraction. The samples were not diluted with Isoton$^{(R)}$ counting fluid, but were counted directly. Counts were corrected with a Coincidence Chart, where necessary.

Culture of Separated Fractions

Fractions to be cultured were determined from a graph of Number of Cells per Fraction versus Fraction Number. If the region of the graph representing cells to be cultured was composed of more than one

fraction, these fractions were poured together to form a pooled group. For example, if fractions 22, 23, 24, 25 and 26 of Experiment 23 were pooled, the pooled fraction group was to be 22/26 (23). For every fraction in the pooled group, 10 ml of Fisher's Medium (10% horse serum + penicillin/streptomycin) were added to the pooled group. The cultures were incubated overnight at 37°C. In the morning, the cultures were centrifuged, the supernatant poured off, and the cells re-suspended in fresh medium.

Karyotypes

Cells to be karyotyped came from two sources.

The lymphoma line was initially karyotyped before any separation experiments were done. During the year that this research was carried out, the line was analysed six times, to determine if any chromosomal shifts occurred. The karyotype has remained constant and is shown in Figure 5. The cells were set up in tissue culture and 0.5 ml of Colchicine (Colcemid$^{(R)}$, Grand Island Biological Company, Berkeley, California) added. The cultures were incubated at 37°C overnight, then karyotyped according to standard procedure.

Cells from separated fractions to be karyotyped were determined and set up in tissue culture according to the method above. 0.5 ml of Colchicine were added to the culture, in the morning after overnight culture of fractions. The cultures were incubated for a further six hours, then karyotyped according to standard procedure.

Standard Karyotype Procedure

The Colchicine-treated cultures were centrifuged, and the super-

natant removed, leaving a pellet containing about 3×10^7 cells. 5 ml of Balanced Salt Solution were added to the cultures which were left to become hypertonic for twenty minutes at room temperature. After re-centrifuging and removal of supernatant, 15 ml of fixative were added, for thirty minutes at 4°C. This step was repeated three times. After the third re-suspension in fixative, the cell solution was assessed for numbers. The number of cells was not so high that the cells were clumped, nor the solution too cloudy. One or two drops of this solution were pipetted on to a slide which was flamed quickly and then air-dried. The slides were then ready for acetic orcein staining. The stain was filtered immediately before use. The slides were left in the stain for 20 - 40 minutes, a time long enough to stain the chromosomes (dark red or magenta) so that they could be seen well with bright field microscopy. The stains were then differentiated in several changes of 95% and absolute ethanol and cover slips applied to the slides with Permount synthetic mounting medium (Fisher Scientific, Edmonton).

The slides were examined under direct illumination with oil-immersion lens, at a magnification of 400X. One hundred chromosome spreads, where possible, were examined. Slides which did not show one hundred spreads (two only) were not rejected, but were analysed on the basis of every chromosome array.

Population Sizing

Cells, freshly removed from the mouse and from 5 day *in vivo*, 1-2 day *in vitro* culture, were measured for diameter size, under Phase Contrast microscopy, at a magnification of 300X, using a Hawksley double-

cell chamber, improved Neubauer haemacytometer (Hawksley, Lancing, Sussex).

The cells were suspended in Fischer's Medium and kept at 37°C before and during sample measurement. 600 cells from each culture were measured and the values entered, sorted and stored in an APL/360 computer (MTS, University of Alberta), through the kind co-operation of Len Friedenberg (Computer Applications to Health Sciences, University of Alberta) and Dr. Tom Overton (Chairman, Biomedical Engineering, University of Alberta).

RESULTS

Determination of the Sedimentation Profile of the Five Day *In Vivo* Cell Population

In order to characterise the five day *in vivo* tumour as to sedimentation profile, five experiments, numbers 27, 28, 29, 30, 32 were performed. Of these five, two experiments, numbers 28 and 29, are not included in the calculation of peak velocities, nor in the discussion of the sedimentation profile. In these two experiments, the profiles showed evidence of 'streaming' (#28) and of the inclusion of many giant and non-viable cells in the starting population (#29). Experiments 28 and 29 are, however, included for discussion of these phenomena.

The following graphs, Figures 6, 7, 8, represent the sedimentation profiles of Experiments 27, 30 32. Each of the sedimentation profiles has a basically unimodal distribution. This is shown in Figure 9 in which the three profiles were averaged to form a single line composite. (This graph will be referred to below as the composite sedimentation profile for a tumour cultured for five days *in vivo*.) The similarity of distribution of the three profiles is shown in Figure 10, in which they are presented in vertical display (common ordinate).

Figures 6 - 10 show the velocity of the 100% cell peak (V_{100}) to be very similar for all three experiments. The average V_{100} is 8.7 mm/hour, with a variance of 0.04 and a standard deviation of 0.2. Table 3 presents the range of V_{100} values and the standard deviation and mean.

In each of the three experiments, a mean of 20.5×10^7 unsedimented tumour cells were loaded into the STAPUT in 60 ml of FCS. Of these cells, an average of 6.8×10^7 cells was collected from the STAPUT. The per cent yield represents the number of cells, collected in all the fractions, expressed as a percentage of the number of cells in the loading sample. This value averaged 43%. The range and mean of these values is given in Table 4.

The results of Experiments 27, 30, 32, therefore show that the sedimentation profile of the five day *in vivo* tumour is unimodal, with a V_{100} value of 8.7 mm/hour. An average of 43% of cells loaded was retrieved from the STAPUT.

Experiments 28 and 29, which were noted as being excluded from the calculations of V_{100} and profile distribution, are presented in Figures 11 and 13. The profile exhibited in Figure 11, (Expt. 28), is the result of 'streaming'. The cells were observed to aggregate into long strings several cells wide, and several centimetres long. Streaming is an aberrant phenomenon, its occurrence related to several factors which were explained in the "Review of the Literature". In Figure 12, an overlay of the 'streaming' profile and the composite profile (Fig. 9) are presented. The shaded areas show the broadening of the profile, as compared to the normal distribution. This occurs most noticeably in the areas between 0 & 8 and 12 & 17 mm/hour. The velocity of the 100% cell peak (V_{100}) is 8.0 mm/hour. The high peak at 0 mm/hour represents the many cells that were 'washed out' of the STAPUT, in the collection of the last fraction. Of a total of 10.8×10^7 cells loaded in 3% FCS, 10.5×10^7 were retrieved, representing a yield of 97%.

The profile presented in Figure 13, Experiment 29, shows the consequences of sedimenting a tumour population containing many non-viable and giant cells. These giant cells were not blast cells (immature, undifferentiated precursor cells) but large cells, with well-vacuolated cytoplasm. A total of 22×10^7 cells were loaded into the STAPUT. Of these, 18×10^6 were non-viable, as shown by the Trypan blue dye exclusion test, and 6×10^6 were giant cells, representing 10.9% of the starting population. In Figure 14, an overlay of this profile and the composite profile (Fig. 9) are presented. Again, as in Figure 12, the shaded areas represent the deviation from the normal distribution. The area of greatest deviation occurs between 14 & 20 mm/hour. The velocity of the 100% cell peak (V_{100}) is 10.0 mm/hour. 44% of the cells loaded were retrieved from the STAPUT. The yield for Experiments 28, 29, and the average for 27, 30, 32 are compared in Table 5.

Thus the results of Experiments 28 and 29 show the consequences of streaming during sedimentation and sedimentation of a partially non-viable cell population.

Determination of the Sedimentation Profile of Cell Populations of Various *In Vivo* and *In Vitro* Time Periods

Group 1: 5 Day *In Vivo*, 1-2 Day *In Vitro* Tumour

To determine if there was any effect on the cell population as a result of time spent in tissue culture, certain tumour samples were not immediately separated in the STAPUT, but were cultured *in vitro* for varying periods of time. The cultures were then sedimented. Four groups of tumour samples were treated this way. Group 1 is composed of tumour cell populations that were removed from the mice after five days of *in vivo*

culture and then cultured *in vitro* for one (Expt. 9) and two (Expt. 7) days. Figures 15 and 16 represent the sedimentation profiles of these experiments. Both the sedimentations have a basically unimodal distribution. There is, in the profile of Experiment 9 [Fig. 15], a small shoulder peak at 5.2 mm/hour. However, comparison with Figure 16 shows the peak to fit within the limits of the profile of Experiment 7. The composite profile is presented in Figure 17, in the form of a single average outline, numerically derived. (This graph will be referred to below as the composite sedimentation profile for a tumour cultured for five days *in vivo* and 1-2 days *in vitro*.) Figures 15, 16, 17 show the velocity of the 100% cell peak (V_{100}) to be very similar for both experiments. The average V_{100} is 6.9 mm/hour, with a variance of 0.10 and a standard deviation of 0.32. These values are presented in Table 6. The similarity of the two profiles, and the difference from the profile of the five day *in vivo* tumour is shown in Figure 18. The three profiles are presented in vertical display (common ordinate).

Group 2: 5 Day *In Vivo*, 1-4 Weeks *In Vitro* Tumour

Group 2 is composed of five day *in vivo* tumours that were then cultured *in vitro* for six (Expt. 24), thirteen (Expt. 25), fifteen (Expt. 21), twenty-one (Expt. 22) and thirty-one (Expt. 23) days. Figures 19, 20, 21, 22, 23 represent the sedimentation profiles of these experiments. Each of the profiles has a basically bimodal distribution. One peak ('fast') occurs in the region of 5 - 12 mm/hour and the other ('slow') between 0 - 4 mm/hour. This is well shown in Figure 24, in which the profiles are presented in vertical display (common ordinate).

At first examination, the V_{100} values for these experiments,

which are given in Table 7, do not show much similarity. The average V_{100} value (sedimentation velocity of 100% cell peak) is 3.2 mm/hour. However, if the two peaks are considered separately, then a more accurate value is achieved. The average for the five 'slow' peaks is 1.9 mm/hour and for the five 'fast' peaks is 6.8 mm/hour. Thus, in this group of experiments, simple consideration of only V_{100} values is not valid.

In each of the five experiments, a mean of 24.5×10^7 unsedimented tumour cells were loaded into the STAPUT in 60 ml of FCS. Of these cells, an average of 22.3×10^7 cells was collected from the STAPUT. The per cent yield represents the total number of cells, collected in all the fractions, expressed as a percentage of the number of cells in the loading sample. This value averaged 88%. The range and mean of these values is given in Table 8.

Therefore, the results of the experiments in Groups 1 and 2 show that *in vitro* culture of the tumour causes changes in the distribution of cell sizes. These changes are reflected in the sedimentation profiles, particularly in the V_{100} values. These values shift from 8.7 mm/hour (0 days *in vitro*), to 6.9 mm/hour (1-2 days *in vitro*), to 6.8 mm/hour ('fast' peak value, 1-4 weeks *in vitro*).

Group 3: 7 Day *In Vivo*, 5 Day *In Vitro* Tumour

In Group 3 are cell populations that were removed from the mice after seven days and then cultured *in vitro* for five days (Expts. 5 & 12), to determine if there was any effect from increased time both *in vivo* and *in vitro*. Figures 25 and 26 represent the sedimentation profiles of these two experiments. Both of the profiles show a basically bimodal distribution. The composite profile is presented in Figure 27, in the

form of a single average outline, numerically derived. (This graph will be referred to below as the composite sedimentation profile for a tumour cultured for 7 days *in vivo* and 5 days *in vitro*.) The two peaks in the profile occur at sedimentation velocity values of 6.8 mm/hour (V_{100}) and 17.3 mm/hour. The latter peak represents the rate of fall attained by cells of about 16.6μ diameter. The 100% cell peak is composed of cells of approximately 10μ diameter, which is the same size as the cells of the V_{100} peak of the 5 day *in vivo*, 1-2 day *in vitro* tumour.

Therefore, the results of the experiments in Group 3 show that the tumour cell population tends to undergo both microcytic and macrocytic changes with increasing time of *in vivo* and *in vitro* culture.

Group 4: Resedimentation of a 7 Day *In Vivo*, 5 Day *In Vitro* Tumour

Group 4 is composed of one experiment (#10) which was run with a cell population cultured *in vitro* for eight days after a previous sedimentation. This original sedimentation (Expt. 6) consisted of a cell population cultured for seven days *in vivo* and five days *in vitro*. The sedimentation profile is trimodal, the three peaks occurring at 16.8, 6.4 (V_{100}) and 1.9 mm/hour. The fastest peak contains cells of 16.4μ diameter while the cells in the 100% cell peak have a mean diameter of 10.1μ. The third or slowest peak is composed of cytoplasmic debris. After sedimentation, fractions 23-29, representing a velocity range of 2.8 mm/hour (5.4-8.2 mm/hour or 100% cell peak), were cultured for eight days *in vitro*, then sedimented, producing the profile shown in Figure 28. This second profile is also trimodal, with peaks of velocities of 8.3, 5.8 (V_{100}) and 1.1 mm/hour. The fastest peak contains cells of 11.5μ

diameter while the cells in the 100% cell peak have a diameter of 9.6μ. The third or slowest peak is composed of cytoplasmic debris. Therefore, the results of the experiments in Group 4 demonstrate the possibility that a small group of separated cells can proliferate and produce an entire size range of tumour cells.

Determination of Cell Diameters by Measurement Under Phase Contrast Microscopy

Having established standard sedimentation profiles for both *in vivo* and *in vitro* cultures, samples of tumour cells were examined for distribution of cell sizes. If the distribution of cell diameters compared favourably with the distribution of sedimentation velocities, then the latter could be assumed to be valid standardisations of the sedimentation profiles.

5 Day *In Vivo* Tumour

Six hundred tumour cells from five day *in vivo* cultures were examined for size range. Diameter values were computed from the micrometer measurements, sorted as to increasing size order, and grouped into one micron sizes. These values were then computed as a percentage of the maximum number of cells in any one group. The largest number of cells was designated 100% and the other values were all computed as a per cent of this maximum (per cent maximum), thus defining a percentile distribution of cell diameters. This distribution is shown in Figure 31, defining a cell size range of 8 to 18μ, with a modal value of 12μ.

To facilitate comparison of the cell size range and the sedimentation profile of the tumour, the cell diameter values were converted

into theoretical (calculated) sedimentation rate values. To convert micron values into mm/hour, the full expression of Stoke's Law was used, rather than the simplification, $s = kr^{2/3}$, to increase accuracy. Thus, in the formula,

$$s = \frac{2gr^2(d_c - d_f)}{9\eta}$$

$g = 981.17$ cm/sec^2, r varies from 8 to 18μ, $d_c = 1.055$ gm/cc, $d_f = 1.004$ gm/cc, and $\eta = 1.72 \times 10^{-2}$ poise (gm/cm/sec) [Kraft, 1972]. (See "Introduction" for explanation of this formula.)

The results of this conversion are plotted on the graph that is Figure 32. Thus, the calculated values represent a theoretical sedimentation profile. Superimposition of this profile onto the measured or actual sedimentation profile for the tumour [Fig. 9] shows the similarity of distribution of the two profiles. Both are unimodal and have identical V_{100} values of 8.7 mm/hour. The calculated profile follows the measured profile but fits within its limits by an average of 1.9 mm/hour.

5 Day *In Vivo*, 1-2 Day *In Vitro* Tumour

Six hundred tumour cells, cultured for five days *in vivo* and *in vitro* for 1-2 days, were similarly examined. The measured values were sorted into whole micron groups and plotted against per cent maximum number of cells [as for Fig. 31]. This second distribution is shown in Figure 33, with a cell size range of 8 to 17μ, mode = 10μ.

Again, as for the five day *in vivo* tumour cells, the measured diameters of the five day *in vivo*, 1-2 day *in vitro* tumour were converted into sedimentation rates (mm/hour). Stoke's Law, with the values given above, was used. The results of this second calculation are pre-

sented in Figure 34. The calculated values represent a theoretical sedimentation profile and are superimposed on a measured or actual sedimentation profile for this tumour [Fig. 17]. The profiles agree well in distribution and the V_{100} values are similar, 6.0 mm/hour (calculated) and 6.8 mm/hour (measured).

Therefore, the results of diameter measurement of *in vivo* and *in vitro* cell populations show a cell size range of 8 to 18μ, modal value = 12μ, and 8 to 17μ, modal value = 10μ, respectively. These diameter distributions compare well with the sedimentation velocity distributions.

Determination of the Karyotype of Separated Fractions of the Five Day *In Vivo* Tumour

Consideration of the theory of the proportionality between cell ploidy and cell size [Hauschka, 1957] prompted chromosomal analysis of various separated fractions of the five day *in vivo* tumour. Fractions from Experiments 27, 30, 32 were subjected to chromosome analysis, to determine the proportion of 2s cells in each fraction. The 2s cells are the double stemline cells, those containing a double complement of chromosomes, but usually with aneuploid duplication. The modal chromosome number for these cells in the tumour studied is 81-82. The modal chromosome number of the s or stemline cells is 42.

The results of these analyses are shown in Table 9. The experiments are listed in order of increasing total time of sedimentation. The velocity (mm/hour) for each "fraction number" represents the average velocity, if two or more fractions were pooled for karyotyping. In Experiment 32, fraction 27-29, the counts were performed on 36 spreads, the total number of spreads available for analysis. In all other cases,

the counts were performed on 100 spreads. The same results are presented in Figure 29, which shows the position of the number of 2s cells on the sedimentation profile of each experiment. The three profiles are arranged vertically, thus showing the distribution of 2s cells, not only for each experiment, but as a comparison among the three experiments.

The results of the chromosome analysis may also be presented directly as a function of the velocity of the karyotyped fraction, rather than as a function of experiment and fraction numbers. Table 10 shows such an ordering. The velocity value given is again the average for the karyotyped fractions, but the range for the fractions included in one analysis is also given.

Organisation of the results of karyotype analysis according to velocity facilitates the designation of a position on the composite sedimentation profile for the five day *in vivo* tumour [Fig. 9] to each 2s value. The velocity values are taken from Table 10 and plotted on the same axis as the profile's velocity values. The percentage of 2s cells is plotted on the ordinate, on a scale twice as large as that of the profile's per cent maximum (number of cells) values. Thus, Figure 30 shows the bimodal distribution of the number of 2s cells in the tumour overlain on the distribution of the sedimentation velocities for the tumour. The two peaks of the 2s cell distribution occur at 9.0 and 11.0 mm/hour. These values represent the rate of fall attained by cells of diameter 12μ and 13.5μ, respectively. Comparison of these diameters to the cell size range for the whole population shows the cells to be of medium size.

Therefore, the results of the karyotype analysis of separated fractions of the five day *in vivo* tumour demonstrate that the distribution of 2s cells does not correspond, as expected, to the distribution of large cells in the population.

DISCUSSION

In this study, a murine ascitic lymphoma, L5178Y, clone FFD, from both *in vivo* and *in vitro* cultures, was characterised as to velocity sedimentation profile. Cells from both *in vivo* and *in vitro* cultures were assigned computed sizes. These calculated diameters were compared with cell diameters of the whole population measured under Phase Contrast microscopy. The cells measured for diameters were also assigned rates of sedimentation (calculated from measured diameters using Stoke's Law). These values constituted calculated (or theoretical) sedimentation profiles which were compared with the measured or actual sedimentation profiles. Cells of separated fractions of the *in vivo* culture were karyotyped to determine the stemline/double stemline chromosome complement distribution.

In the first part of the discussion the results will be interpreted. In the second part, the methods used, velocity sedimentation and diameter measurement, will be evaluated. However, before commencing this discussion, there are two comments of general importance. First, there is some degree of biological variability among the tumour samples used, mostly among the 5 day *in vivo* tumours. Due to host factors, each tumour sample will tend to be slightly different, even to a sister-cell tumour from the same parent tumour and innoculated in the host at the same time. For example, the number of normal lymphocytes found within the tumour may vary and it was not possible to make any correction for this occurrence. However, in working with *in vivo* populations it is assumed that there will

be some degree of variability and the results may be interpreted as presented. Also, because none of the sedimentation profiles represent normal distributions, no statistical analyses of the profiles (t-test, Chi-square) were performed.

Interpretation of Results

Microscopic examination of the tumour, prior to any experiments, had shown a large variance in cell size. Despite the fact that tumour cells show great differences in cell size, it was thought that perhaps three distinct sub-groups, (based on cell size classification of small, medium, and large), could be demonstrated in the population. These three sizes would correspond to pre- and post-mitotic and resting stages of the cell cycle. A factor which further supported this idea was the tumour's chromosome complement. L5178Y, clone FFD, is 70% stemline, 30% double stemline. Consideration of the fact that increase in number of chromosomes should require an increase in cell size suggested that there should be at least two distinct cell sizes in the population, small stemline and large double stemline cells. Thus, the sedimentations were planned in the hope of demonstrating two, if not three, sub-groups in the population.

5 Day *In Vivo* Tumour

Three sedimentations were performed on cells cultured for five days *in vivo*. The profiles, all of which represent unimodal distributions, do not show any evidence of sub-groups within the population. The profiles could, of course, as a result of technical difficulties, be considered invalid representations of the sedimentation distribution and

therefore of the cell size distribution. Because of this, cell diameters for this tumour were measured under Phase Contrast microscopy. The distribution of cell diameters is also unimodal. To compare this distribution with the sedimentation profile, the micron values were converted into mm/hour, using Stoke's Law. Thus, a calculated (theoretical) profile was overlaid on a measured (actual) profile. The profiles compare extremely well, having identical modes, and the calculated profile following the measured profile, but fitting within its limits. This slight quantitative difference might be due to the fact that only viable cells were measured for diameter sizing. (Cytoplasmic debris and non-viable cells were observed but were not measured.) Similar populations were sedimented and therefore the presence of any debris, etcetera, would be indicated on the profile. Or, the difference might be due to the fact that one density value was assumed for the tumour cells, in the velocity calculations. A slight density difference between, for example, large and small cells, would result in a velocity difference, in comparison to the theoretical sedimentation profile. Also, although the V_{100} values compare extremely well, the calculated sedimentation rate for the diameter-sized cells is just that. Cells of 10μ diameter, although calculated to sediment with a velocity of 6 mm/hour, may actually sediment at 5 mm/hour (value taken from comparable position on measured sedimentation profile), perhaps again as the result of density differences. Thus, the measured and calculated sedimentation profiles compare extremely well, establishing the sedimentations of the 5 day *in vivo* tumour as valid separations.

5 Day *In Vivo*, X Day *In Vitro* Tumours

To determine the effect of tissue culture on the tumour, sedimentation studies were carried out using tumours cultured *in vitro* for varying periods of time.

Experiments 9 and 7 were run with 1-2 day *in vitro* cultured tumours. Both profiles have basically unimodal distributions, thus representing unimodal cell size distributions. However, the existence of a small shoulder peak in the profile of Experiment 9, which fits within the limits of the profile of Experiment 7, suggests the possibility of a subgroup of cells (calculated diameter approximately 9μ). Examination of the calculated profile for this tumour shows no similar distribution in sedimentation rates (and therefore in cell diameters). (The calculated profile was determined from the distribution of measured cell diameters, as for the 5 day *in vivo* tumour.) Both the diameter and calculated velocity distributions are unimodal and the calculated and measured sedimentation profiles also agree well, having very little difference in V_{100} values. (What little difference there is could again be explained by density differences.) It is therefore possible that the presence of this small peak is an aberration. The cells were sedimented after only one day in tissue culture and the probability of the population reacting to the new environment (thus stimulating formation of a group of smaller cells) is not negligible.

The suggestion of a reaction to a change in environment (from *in vivo* to *in vitro*) is not inconceivable. The tissue culture system, although well designed for optimal growth of these cells, can obviously not duplicate the many host factors that influence the cells. This is

well shown by Figures 18 and 35. In Figure 18, three sedimentation profiles, the composite for the 5 day *in vivo* tumour, Experiment 9 (1 day *in vitro*) and Experiment 7 (2 days *in vitro*), are presented in vertical display. This display shows the shift in profile distribution that occurs with change in *in vitro* culture time, from 0 to 1 to 2 days. In Figure 35, the composites for the 5 day *in vivo* tumour and the 5 day *in vivo*, 1-2 day *in vitro* tumour are compared. The V_{100} values differ by 1.9 mm/hour which means that the modal cell size could differ by about 2μ (diameter). The profile for the 1-2 day *in vitro* tumour is shifted to the left of the other profile, thus indicating the presence of fewer large cells and more small cells in the population. This microcytic response, perhaps due to nutritional differences between the two environments, also occurs in blood: microcytic (hypochromic) anaemia results from nutritional iron deficiency [Weatherall, 1967]. However, the microcytosis might not be an aberrant response to tissue culture but rather part of the progression of the tumour cell population. The tumour sedimented in Experiment 9 had been cultured for a total of six days. The small shoulder peak could represent a sub-group of cells not found before five days of tumour growth. The sub-group would consist of small cells (9μ diameter), some of the progeny of perhaps the 12μ cells (modal cell size of the 5 day *in vivo* profile and decreased in number by 50% in the 1 day *in vitro* profile). The microcytic shift could then be part of a small cell (post-mitotic) - large cell (pre-mitotic) cycle. This would be evident in the tumour only after at least six days of culture, rather than being an aberrant response to the conditions of *in vitro* culture. The small cell peak is not distinct in the profile of the 2 day *in vitro*

tumour (seven days of culture). This suggests the possibility that these cells are growing and the range (distribution) of increased cell sizes precludes separation of any particular cell size. Further sedimentation or re-sedimentation of a few fractions would be required to define the origins of the peak. (The shift in cell size is further discussed below, in the section "7 Day *In Vivo*, 5 Day *In Vitro* Tumour".) Thus, sedimentation of a 5 day *in vivo*, 1-2 day *in vitro* tumour shows the microcytic response induced in the tumour either by the shift from the *in vivo* to the *in vitro* environment or as a result of tumour progression.

Experiments 24, 25, 21, 22, 23 were run with tumours cultured for 6, 13, 15, 21, 31 days *in vitro*, respectively. Obviously, the use of tumours so widely variant with respect to culture time would tend to cause dissimilarity of the profiles. With the exception of Experiment 23, the distributions are bimodal. The V_{100} values do not coincide, but have a wide range, making calculation of a mean V_{100} value difficult. (The standard deviation becomes very large if all five values are included.) Yet the profiles do not differ too much, for overlay comparison shows general agreement of the two peaks present. The base of the peaks in all five cases occurs between 5 & 12 mm/hour and 0 & 4 mm/hour. Thus, there is general agreement among the positions of the 'fast' and the 'slow' peaks, the latter having a mean rate of fall usually attributable to cytoplasmic debris. The 'fast' peak value shows a decrease with increasing *in vitro* culture time, suggesting either a reaction to the tissue culture system or evidence of tumour development, as explained above. The profile of Experiment 23 is trimodal and broad but the peaks are distinct enough to suggest three sub-groups within the population sedimented. This change

in population make-up and the shift from two clearly separated peaks to three fairly distinct peaks is well shown in Figure 24. The profiles are presented in vertical display, showing the change in relative heights, but similarity of distribution, of the major peaks. Thus there is a definite change in the cell population, the longer the tumour is cultured (*in vitro*). Although the cells continued to grow at maximum rate, requiring a change of medium every day, it is possible that there was some deficit in the *in vitro* system for these cells. This deficit could be nutritional, such as a lack of an essential amino acid or an overabundance of a precursor, thus causing, through a negative feed-back system, a lack of a metabolic by-product. Or the deficit could be cellular in that the lymphoma cells might require some sort of feeder cells, either for metabolite fabrication or for removal of a by-product, inhibitory above a certain level. Several experiments are therefore needed to determine the exact requirements of this population. One interesting, correlative fact is that the clone FFD can only be grown for about a month in tissue culture before deterioration of the population (polyploidy, absence of growth) occurs. Experiment 23 was a 31 day *in vitro* culture and this might explain the presence of three sub-groups within the population. Therefore, the results of these experiments show that tumour populations cultured *in vitro* for periods of one to four weeks tend to undergo microcytosis and then not to grow well, to the point of partial degeneration of the population.

7 Day *In Vivo*, 5 Day *In Vitro* Tumour

Observation of the cell populations' microcytic and degenerative

responses to increased time *in vitro* prompted sedimentation of a tumour cultured for a longer period of time *in vivo* and *in vitro*. Experiments 5 and 12 were run with 7 day *in vivo*, 5 day *in vitro* tumours. Both profiles are definitely bimodal distributions. Comparison of the composite profile with that of the 5 day *in vivo* tumour [Fig. 36] demonstrates not only the appearance of the second peak, representing large cells of about 16μ diameter, but also the shift to the left (slow region of the profile) of the major (100%) peak. This shift represents a probable difference of 2μ between cell diameters of the two tumour populations at this region. This is the same as the difference between the profiles for the 5 day *in vivo*, 1-2 day *in vitro* tumour and the 5 day *in vivo* tumour. In fact, comparison of the 7 day *in vivo*, 5 day *in vitro* tumour (12 days culture) and the 5 day *in vivo*, 1-2 day *in vitro* tumour (7 days culture) profiles shows great similarity. Except for the presence of the second peak (large cells) in the former profile (12 days culture), the distributions coincide well, having identical V_{100} values. This suggests that the appearance of the large cell peak in the 12 day culture is the result of the extra five days in culture. Both cultures were grown *in vitro* and therefore there should be no differences as a result of, for example, environmental change. Seven days of culture of both populations produced proliferation of smaller cells in the modal region of the peak, in comparison to cell sizes of the five day *in vivo* culture. Thus, the extra days *in vitro* (12 total) led to the appearance of a peak of large cells. These cells, as explained above, are probably pre-mitotic (large) forms. The timing of their appearance may be related to rate of growth of the small cells from which the large cells derived. (The large cells, on division,

would continue to feed the peak of small cells, which would grow and therefore cyclically replace the large cells.) Thus, the results of these experiments show that increased time of culture (*in vivo* and *in vitro*) tends to cause microcytic, followed by macrocytic, responses in the tumour cell population.

Resedimentation of a 7 Day *In Vivo*, 5 Day *In Vitro* Tumour

Experiment 10 was run with fractions from a previous sedimentation (Expt. 6). The original sedimentation used a tumour cultured for 7 days *in vivo* and 5 days *in vitro*. The profile of this sedimentation is trimodal, representing cells of 16.4μ and 10.1μ (V_{100}) diameter and cytoplasmic debris. These results are in good accordance with those of sedimentation of similarly cultured tumours (Expts. 5 & 12). The cells of the V_{100} peak were cultured *in vitro* for eight days, and the entire culture then sedimented (Expt. 10). This second profile is also distinctly trimodal, with peaks representing cells of 11.5μ and 9.6μ (V_{100}) diameter and cytoplasmic debris. Because of the length of time in tissue culture (13 days), this profile cannot be exactly compared with others. However, examination of the profile does provide interesting information about the cells in the 100% peak region of Experiment 6. The profile for Experiment 10 shows a full sedimentation velocity range, thus being composed of cells ranging from at least 8 to 18μ in diameter. Yet, these cells are the progeny of cells of diameter range of only 9.3 to 11.5μ. These latter cells are therefore capable of producing an entire range of tumour cells. This would suggest that these cells are some form of stem cell, capable of differentiating and/or dividing to produce a complete popula-

tion. Or, if not such a basic form, then these cells are perhaps a middle stage in normal tumour proliferation, representing a growth stage, derived from small cells (post-mitotic), and on the way to becoming large cells (pre-mitotic). Either of the two theories is probable. The latter is reminiscent of growth patterns of normal cells, which these are not. The former, however, is given some weight by the theory of differences between sister cells of the same tumour. Cancer cells produced by a single primary tumour vary. Recent studies have shown that "individual cells producing metastases (are) ... intrinsically different", despite a common tumour origin, perhaps as a result of the "varying effect of carcinogenic agents on individual cells at a target site" [Delarue, 1972]. Thus, those cells isolated in the 100% cell peak of Experiment 6 could very well be the basic clone of tumour cells, capable of differentiating to produce the many different types of neoplastic cells. Further experiments, to test oncogenicity (and immunogenicity) of different fractions of cells, are therefore strongly suggested. This testing would involve grouping of fractions of cells on the basis of sedimentation velocity, and therefore on the basis of cell size.

Karyotypes of Separated Fractions of the 5 Day *In Vivo* Tumour

Sedimentations were planned in the hope of separating at least two sub-groups of cell sizes. These two groups would be composed of small or stemline cells and large or double stemline cells, the theory being that increase in cell ploidy demanded an increase in cell size. This theory was based on work by Fankhauser, Briggs, and recently, Hauschka and co-workers.

Fankhauser's original study was on the effect of changes in chromosome number on amphibian development. He measured the size of the interphase nuclei of epidermal cells, which being flat discs facilitated observation. The nuclear size increased "roughly in proportion to the number of chromosomes" which the cells contained, not only in the shift from 2N to 3N but from 3N to 5N. Although this increase was only in nuclear size, Fankhauser determined that the whole cell size increased, because body size of the embryos remained constant and total number of cells decreased [Fankhauser, 1945]. Two years later, Briggs reported that the ectodermal cells of 3N frog embryos were "definitely larger than the corresponding cells of the diploid" by 1.5 times. But the embryos grew at the same rate as the 2N, thus confirming Fankhauser's findings [Briggs, 1947].

Fankhauser's second study concerned nucleo-cytoplasmic relations in amphibian development. He noted that the "graded increase in nuclear and cell size in amphibian embryos with varying degrees of polyploidy" might be due to the control of a "definite nucleo-cytoplasmic ratio". First, the nuclei increased their size in direct proportion to the number of chromosome sets. Then, the larger nuclei caused an increase in total amount of cytoplasm, this "mass effect" therefore being under genetic control [Fankhauser, 1952]. Five years later, Hauschka and co-workers demonstrated a "close correlation between chromosome ploidy and cell volume in ... cancer tissue". This was shown by "agreement between the ratios for average DNA content per cell and average cell volume" [Hauschka *et al.*, 1957].

Thus, there developed the concept that those cells which were polyploid would be larger in volume than the diploid cells. Larger volume requires larger diameter size and therefore diploid or stemline cells should be separable from tetraploid or double stemline cells on the basis of size. Also, the observation that the numbers of chromosomes increase as cells become malignant [Schmid & Hutchison, 1972] suggested that the larger cells of this tumour might possess a chromosome complement more directed to neoplasia than the smaller cells. Successful separation would then permit further studies of *in vivo* and *in vitro* tumourigenicity.

Chromosomal analysis of certain separated fractions of the 5 day *in vivo* tumour shows a bimodal distribution of 2s cells, at 9.0 and 11.0 mm/hour. The former peak agrees quite well with the mode of the tumour composite profile. The second peak is skewed 2 mm/hour to the right. Expecting the tumour population to follow the rule of cell ploidy determing cell size suggested that the 2s cells all distribute in the 'fast' region of the profile, between 15 & 17 mm/hour. Instead, the cells distribute partly in a 'slow' velocity region, corresponding to a range of about 9.2 - 12μ diameters. Yet the smallest cells of the population are only 8μ diameter. The cells are also found in a mid velocity region, corresponding to a cell size range of 12.1 - 13.5μ, definitely medium-size cells in a population that contains cells of up to 18μ diameter.

At first examination, therefore, these results are in direct disagreement with those of Fankhauser, Briggs, and Hauschka and co-workers. The amphibian results could be accounted for by a criticism of the techniques of Fankhauser and Briggs. Cell sizes were determined from microscopic examination of "whole mounts of tail tips clipped from living

larvae soon after hatching", a difficult procedure, especially as the "boundaries (of the cells were) usually not clearly marked" [Fankhauser, 1945]. Yet, their results should be accepted, for other observations (such as decrease in the number of cells of the lateral line sense organs in polyploid frogs) suggest verity. The experiments of Fankhauser and Briggs were, however, all conducted on basically artificial material. The polyploidy embryos were produced by subjecting the fertilised eggs to temperature shock. Fankhauser himself states that, in many plant and animal tissues, increase in ploidy does not alter nucleo-cytoplasmic ratios [Fankhauser, 1952]. Thus, this 'natural' tumour may reflect more the true state of ploidy-size relations, than do the artificial amphibians.

The results of Hauschka and co-workers were derived from a comparative study of two populations, a hyperdiploid line (ELD, modal chromosome number = 45 - 46) and a hypertetraploid line (ELT, modal chromosome number = 90 - 92). These two lines were grown separately and this introduces the interesting question of whether these cells would have grown this way had they been mixed in culture. "In organs and tissues in which the size and shape of cell populations are controlled, the replicating cells must have information of the number and position of cells in the population" [Loewenstein, 1969]. One of the factors which determines cell position is cell size. Therefore, it is not inconceivable that the presence of both hyperdiploid and hypertetraploid cells in the same population exerts some sort of controlling factor over their growth, and thus size of these cells. One further point is that as long as all genes are present in the same numbers, "whole sets of genes may be multiplied without disturbing the harmonious activity of the gene complex"

[Fankhauser, 1952]. Conversely, the presence of partial sets of genes may cause differences in genetically controlled activities. Both the L5178Y, ELD and ELT tumours are hyperdiploid and hypertetraploid. Allowing for the "relatively greater influence on nuclear size" (and therefore on cell size) of some chromosomes than of others, [Fankhauser, 1945], the different mass effects are explainable.

Therefore, the results of chromosomal analysis of separated fractions of the 5 day *in vivo* tumour fail to show that, for this tumour, cell ploidy does not determine cell size. This disagreement with the ploidy-size theory may be explained as the result of genetic and environmental influences on the tumour cells. However, re-examination of the ploidy-size theory, using current methods of cell separation, is suggested.

Evaluation of Methods

Velocity Sedimentation

The technique of velocity sedimentation has been in use for about six years now. Since the technique's inception, scientific opinion has changed from regarding cell separation at unit gravity as an impossibility [Agranoff *et al.*, 1954] to recognising it as a standard method of cell analysis. Yet, the velocity sedimentation system is, like other cell separation methods, subject to criticism with respect to the validity of the actual separation. One may question as to which elements have been separated (from a starting population or from each other) or if some separated components are simply artifacts of the method used.

For example, the results of Experiment 29 were not included in the calculation of results with the other experiments using 5 day *in vivo*

cultures because of the nature of the starting population in Experiment 29. This tumour population contained many giant and non-viable cells, representing 10.9% of the starting population. Comparison of this sedimentation profile with the composite profile for this tumour shows a single 6 mm/hour wide region of broadening of the profile occurring in the former but not in the latter profile. Calculation of the per cent of cells found in this region shows 14.8% cells to be present here. This suggests that the giant and non-viable cells were responsible for this unilateral broadening of the profile. Further weight is given to the theory by consideration of the size of these cells. Giant cells are, obviously, large, but so also are damaged cells, which tend to absorb H_2O and ions. (This is the mechanism of the Trypan blue dye exclusion test.) If the population had not been well examined before separation, identification of the cells in this area could have been difficult, perhaps leading to the erroneous assumption of the existence of a sub-population not quite separated from the main peak of cells. Thus, in this case, the separation was no doubt valid, but confusing at first as to which elements had been separated.

A second example is given by Experiment 28. The results of this experiment were also not included for calculation with the other 5 day *in vivo* tumour sedimentations because of the presence of 'streaming'. As explained in the section "Review of the Literature", there are two causes of streaming. These are overloading of sample layer beyond the streaming limit (number of cells/ml of loading volume) and poor mixing of cells within the solute [Mel, 1964b]. That streaming did occur is best shown in Figure 12, an overlay of the streaming profile onto the composite pro-

file for the tumour. In this case there is bilateral broadening of the profile showing streaming (in contrast to the unilateral broadening of the profile in Experiment 29). When streaming occurs, cells are carried down in strings, to regions where they would not normally be found. Also, as these cells, which have "ceased to sediment as independent entities" and "now move in groups", travel through the gradient, they carry with them an "amount of entrapped liquid" [Mel, 1964b]. This causes the production of upward columns of liquid, equal in amount and in rate of flow to the downward streams. The upward streams of liquid then carry other cells toward the top of the gradient. Thus, the cells will be more widely dispersed through the gradient, leading to the bilateral broadening of the profile. Such broadening could actually be interpreted as representing a multicomponent population, of individual sub-units separable after repeated sedimentation. Thus, in this case, such interpretation would be due to an artifact of the separation procedure.

Unhealthy starting populations and the occurrence of streaming are two factors which, if present, should lead to questioning of the validity of the separation. A third factor is low yield of cells retrieved from the STAPUT after sedimentation. For example, in Expts. 27, 30, 32, the mean yield of cells was 43%. Therefore, an average of 57% of the cells that were loaded into the STAPUT were not retrieved. This 'loss' of more than one-half the cells loaded poses two questions. First, what happened to these cells? Second, what does this 'loss' mean to the validity of the separation?

First, the 'missing' cells probably had two fates. In Figure 7,

(Experiment 30), there is a large peak (35%) at 0 mm/hour. This peak represents 'wash-out', or cells that were 'washed-out' of the STAPUT in the collection of the last fraction. 'Wash-out' may occur when flow rates during sampling are so low that cells escape the meniscus of the fluid and are left behind on the walls of the STAPUT. This occurs most easily in the cone section of the STAPUT, where the sloping walls reduce the value for drop in vertical height/unit time. (Obviously, at a constant rate of flow, descent of the fluid meniscus per unit time in the cylindrical section is going to be greater than in the conical section in which there is very little change in the vertical.) Now, comparison of the Collection time (T_c) for Experiment 30, with 35% 'wash-out', and for Experiment 27, with only 17% 'wash-out', shows identical T_c times of 36 minutes. Thus, a too-low rate of sample collection is not indicated as the cause of this 'wash-out'. The phenomenon of 'wash-out' may also occur if certain factors promote the sticking of cells to the walls of the vessel. Cells, especially those which grow in monolayer in culture, or cancer cells which tend to be 'sticky', will adhere to glass unless the surface is coated with silicone. But, as mentioned in "Materials and Methods", the STAPUT was siliconised before each experiment. The only logical explanation is that the cells used in Experiment 30 were somehow more 'sticky' than those used in, say, Experiment 27. This is therefore a question of biological variability of the starting population as sometimes occurs with such use of a "system involving long-transplanted tumours" [Schmid & Hutchison, 1972]. For example, in 1958, only 10 cells of L5178Y were needed to cause "leukaemia when injected into mice of the strain of origin" [Fischer, 1958]. Fourteen years later, the tumour

has altered to the point that an intraperitoneal "innoculum of 100 cells of L5178Y is not lethal" [Schmid & Hutchison, 1972].

To return to the 'missing' cells, 'wash-out' could account for the loss of some cells. Other cells might remain on the sides of the STAPUT (not 'washed-out'). However, the second possibility accounting for 'loss' of cells is that the cells are not 'missing'. Yield of cells is calculated from the number of cells loaded into the STAPUT. Therefore, if an error is made in either counting or dilution of cells at the start of the experiment, this error will influence yield calculations. For example, in the calculation of the yield of cells retrieved from the STAPUT for Experiments 21-25, the yield for Experiment 21 was 137%. In the velocity sedimentation system, it is impossible to retrieve more cells than were put in. Operation at 4°C precludes mitosis (beyond the 1% level of cells that were just completing telophase). Thus, for this experiment, some error was made at the start, either in counting or dilution of cells. (There is also the possibility that this error also occurs in the opposite direction. In Experiment 24, for example, the yield was only 58% and in Experiment 27 only 17%. Thus, a very low yield might be the result of cell-preparation error, rather than actual sedimentation experiment error.)

Having offered explanations for how cells may be 'lost' during an experiment, the effect of this 'loss' on the validity of the separation must now be considered. Clearly, if only one-half the starting population is available for examination at the end of the experiment, then the results have a 50% possibility of being valid. The cells 'lost' may represent exactly one-half of all components of the population. Thus, the

'loss' concerns actual numbers only and therefore the results of separation should be considered valid. Or, the 'loss' may be selective, removing only certain components from the population and leaving others in full number. Thus, the separation is an artifact of the method and the results are invalid. However, there is no way to determine which of the two explanations is correct and all results will be considered valid.

No mention of lack of reproducibility of the method of velocity sedimentation was made in any of the papers studied during the course of the review of the literature. Furthermore, results of separation have agreed with those achieved by use of other methods, for example, density gradient centrifugation [Haskill & Moore, 1970]. In the experiments reported above ("Results"), good agreement was obtained among sedimentations using similar culture populations, despite differences in length of time of experiment. Thus, velocity sedimentation represents a very reproducible and valid method of cell separation.

Diameter Sizing

The technique used for measurement of diameters is the standard one for determining cell size. The method is limited by requiring an accurate reading from a micrometer scale, but this is facilitated by the use of Phase Contrast illumination. Thus, all the viable cells present as spheres, ringed black. Counting of only viable cells does, of course, tend to give a slightly distorted view of the whole population, which is normally composed of viable, non-viable cells and cytoplasmic debris.

CONCLUSIONS

The murine ascitic lymphoma, L5178Y, clone FFD, from both *in vivo* and *in vitro* cultures, was characterised as to velocity sedimentation profile.

(1) Sedimentation profiles of tumour cultured *in vitro* for increasing periods of time showed evidence of micro- and macrocytic changes in the cell population. These changes were postulated to be either part of tumour progression or a reaction to environmental change (*in vivo* to *in vitro*).

(2) Cells, grown *in vitro* from the 100% cell peak (medium-sized cells) of a previous sedimentation and then re-sedimented, showed a full complement of cell sizes in the profile. These medium-sized cells may have a stem-cell function.

(3) Cells of separated fractions were assigned computed sizes. Calculated cell diameters compared well with those of the whole population measured under Phase Contrast microscopy. The cells measured for diameters were also assigned rates of sedimentation. These values constituted calculated (or theoretical) sedimentation profiles, and again compared well with the measured (or actual) sedimentation profiles. Validity of the separation profiles was therefore established.

(4) Karyotypes of cells of separated fractions from 5 day *in vivo* tumours showed a bimodal 2s cell distribution, the cells having sedimentation velocities corresponding to those of medium-

sized cells. These results fail to agree with the increase in cell ploidy, increase in cell size theory, which implied that the 2s cells would be large. Re-examination of the theory is suggested, using current cell separation methods.

TABLES

Table I Phosphate Buffered Saline, according to Dulbecco & Vogt (1954)

Solution A		Solution B		Solution C	
NaCl	8.00 gm	$CaCl_2$	0.10 gm	$MgCl_2 . 6H_2O$	0.10 gm
KCl	0.20 gm	H_2O	100 ml	H_2O	100 ml
Na_2HPO_4	1.15 gm				
KH_2PO_4	0.20 gm				
Water to 800 ml					

Table 2 Solutions for Karyotyping

Hypertonising Balanced Salt Solution

1 ml Earle's Balanced Salt Solution

4 ml distilled H_2O

Fixative Solution

3 parts Methanol

1 part Acetic Acid

Table 3 Velocity of 100% Cell Peak (V100) (mm/hour) (5 day *in vivo* tumour)

Experiment #	Velocity of 100% Cell Peak (V100) (mm/hour)
27	8.7
30	8.9
32	8.5
AVERAGE	8.7 (Variance = 0.04, S.D. = 0.20)

Table 4 Yield of cells retrieved from STAPUT (Expts 27, 30, 32)

Experiment #	# Cells In	# Cells Out	% Yield
27	30.0×10^7	5.2×10^7	17
30	9.6×10^7	7.3×10^7	76
32	22.0×10^7	7.9×10^7	36
AVERAGE	20.5×10^7	6.8×10^7	43

Table 5 Comparison of per cent yield of cells among sedimentations of 5 day *in vivo* tumour

Experiment #	Total % Yield	Comments
27, 30, 32 (Av)	43	Normal
28	97	'Streaming'
29	44	Giant & non-viable cells

Table 6 Velocity of 100% Cell Peak (V100) (mm/hour)
(5 day *in vivo*, 1-2 day *in vitro* tumour)

Experiment #	Velocity of 100% Cell Peak (V100) (mm/hour)
9	6.7
7	7.1

AVERAGE 6.9

(Variance = 0.10,
S.D. = 0.32)

Table 7 Velocity of 100% 'Slow' & 'Fast' Cell Peaks (mm/hour) (5 day *in vivo*, 1-4 weeks *in vitro* tumours)

Experiment #	Days in vitro	100% Cell Peak (mm/hour)	'Slow' Peak (mm/hour)	'Fast' Peak (mm/ hour)
24	6	7.4	1.4	7.4
25	13	2.1	2.1	7.2
21	15	1.0	1.0	6.8
22	21	2.2	2.2	6.2
23	31	2.9	2.9	6.4
AVERAGE	--	3.2	1.9	6.8

Table 8 Yield of Cells retrieved from STAPUT
(Expts. 24, 25, 21, 22, 23)

Experiment #	# Cells In	# Cells Out	Total % Yield
24	5.0×10^7	2.9×10^7	58
25	30.0×10^7	21.9×10^7	73
21	22.5×10^7	30.8×10^7	137
22	15.0×10^7	12.9×10^7	86
23	50.0×10^7	42.8×10^7	86
AVERAGE	24.5×10^7	22.3×10^7	88

Table 9 Karotype analysis of separated fractions of five day *in vivo* tumour, (presented according to experiment and fraction number)

Experiment #	Fraction #	Velocity (mm/hour)	%S	%2s
27	23 - 24	11.0	77	23
	25 - 28	9.0	91	9
30	24	11.5	93	7
	27 - 28	9.2	99	1
32	1 - 13	15.5	71	29
	14 - 17	11.0	66	34
	18 - 19	9.6	98	2
	20 - 24	8.1	97	3
	25 - 26	6.6	99	1
	27 - 29	5.5	100	0

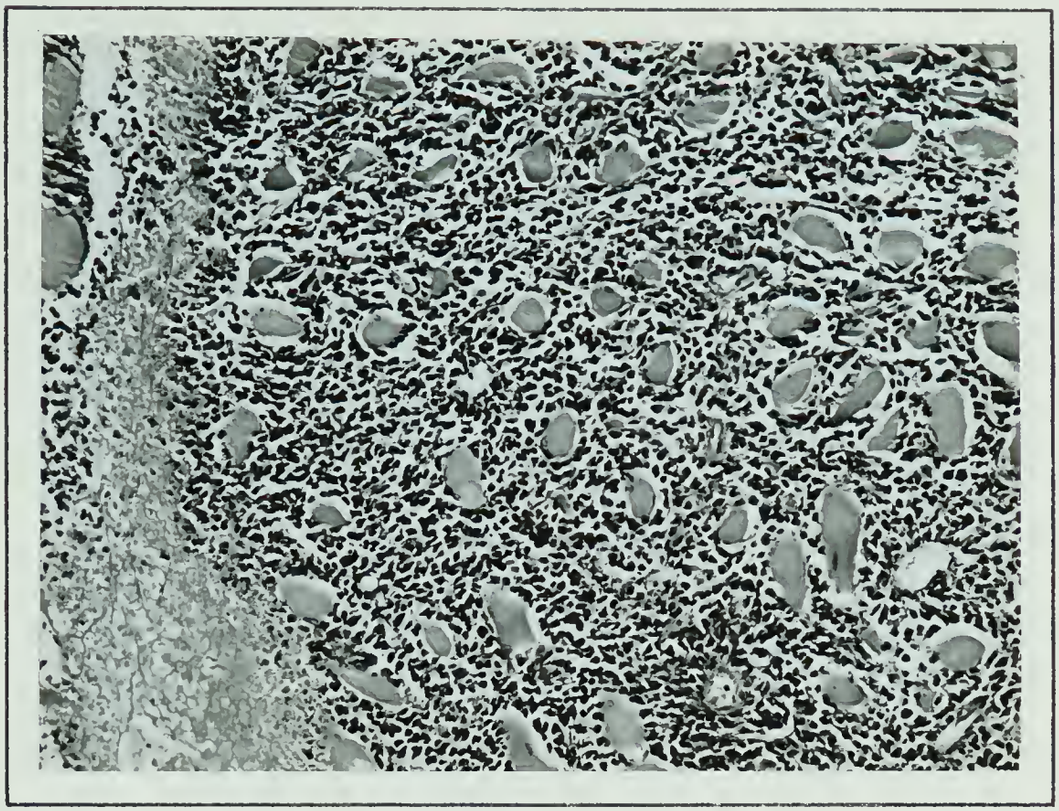
Table 10 Karyotype analysis of separated fractions of five day *in vivo* tumour (presented according to velocity [mm/hour])

Velocity (mm/hour)	Range of Velocities of Karyotyped Fractions (mm/hour)		%2s
5.5	5.9 - 5.1	0.8	0
6.6	6.8 - 6.3	0.5	1
8.1	8.9 - 7.2	1.7	3
9.0	10.0 - 8.1	1.9	9
9.2	9.5 - 8.9	0.6	1
9.6	9.9 - 9.4	0.5	2
11.0	11.4 - 10.7	0.7	23
11.0	11.8 - 10.3	1.5	34
11.5	-- --	--	7
15.5	18.7 - 11.8	6.9	29

FIGURES

Figure 1 Histological section of L5178Y tumour in BDF_1 mouse at one week (125X)

Figure 2 Smear of L5178Y tumour in BDF_1 mouse at one week (125X)



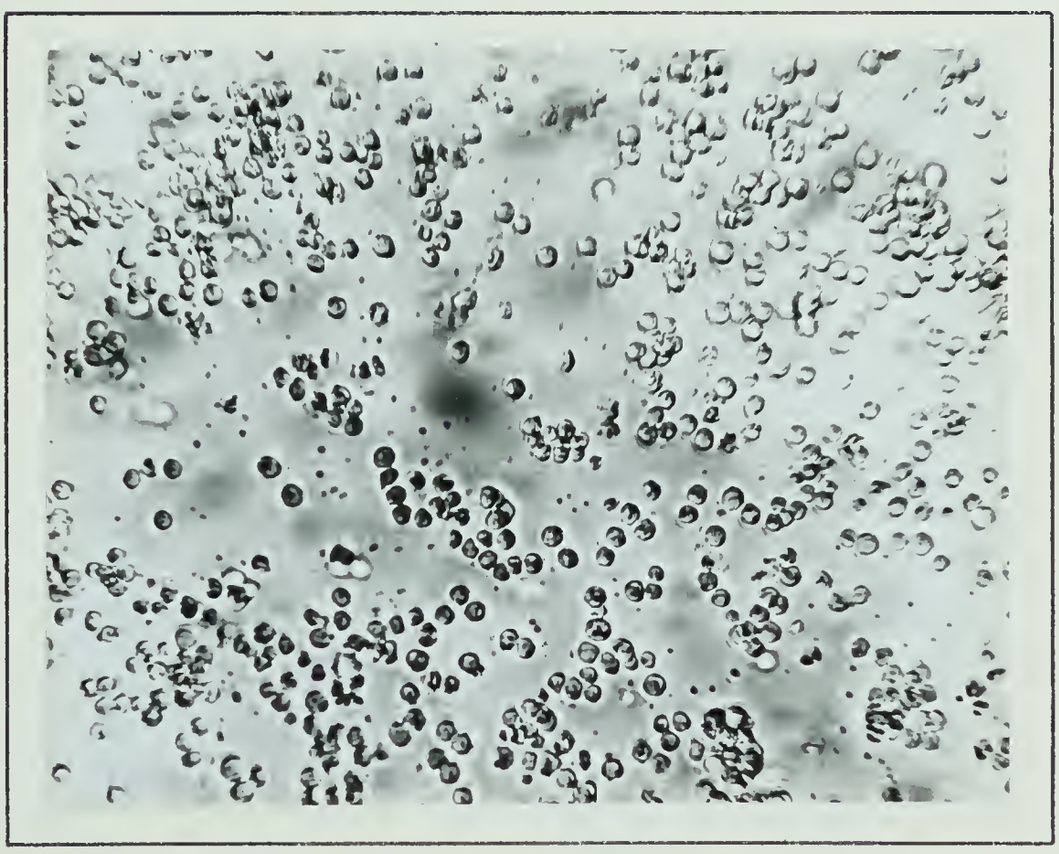


Figure 3 Experimental Protocol

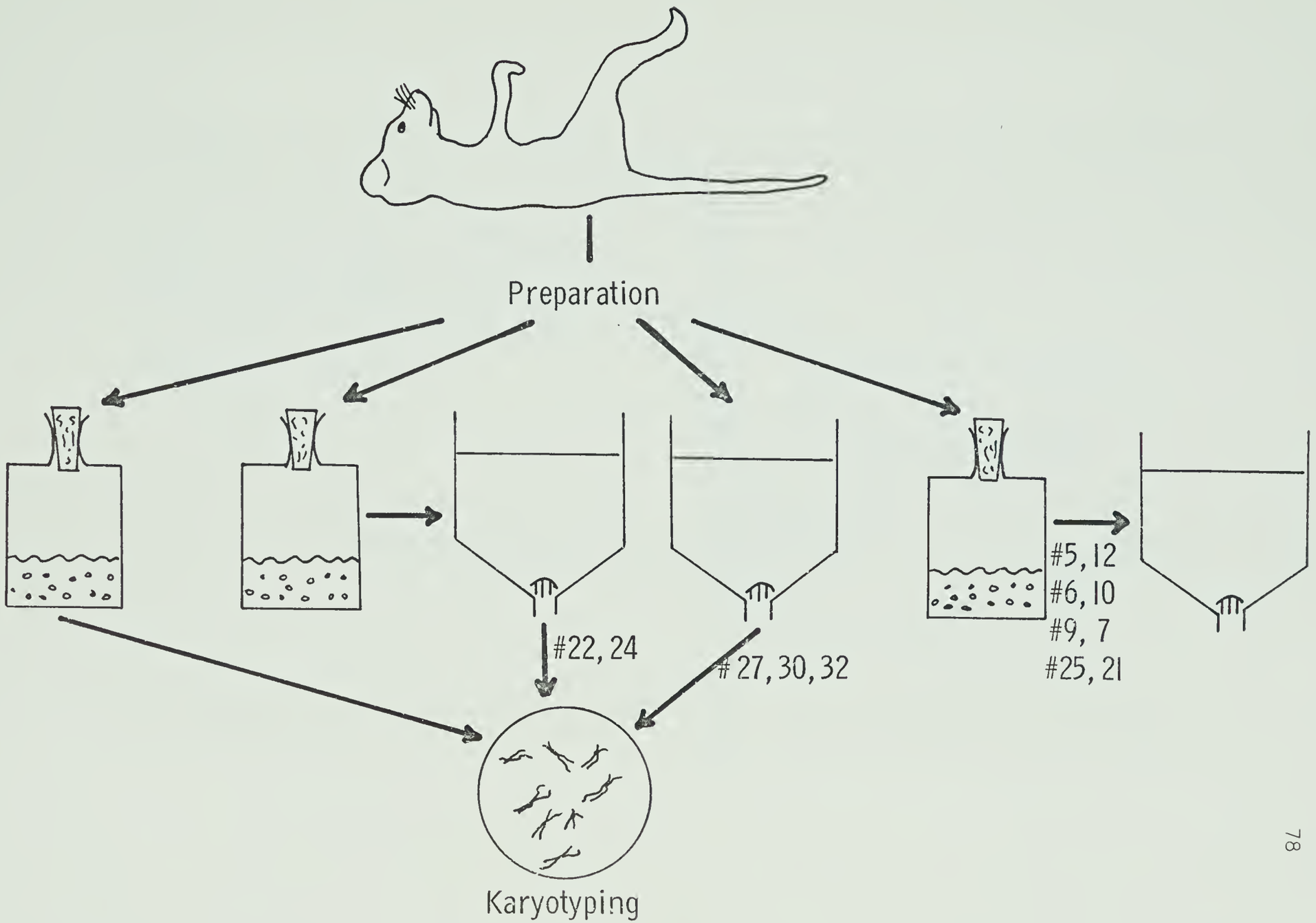

Preparation
#22, 24
#27, 30, 32
#5, 12
#6, 10
#9, 7
#25, 21
Karyotyping

Figure 4 Velocity Sedimentation System

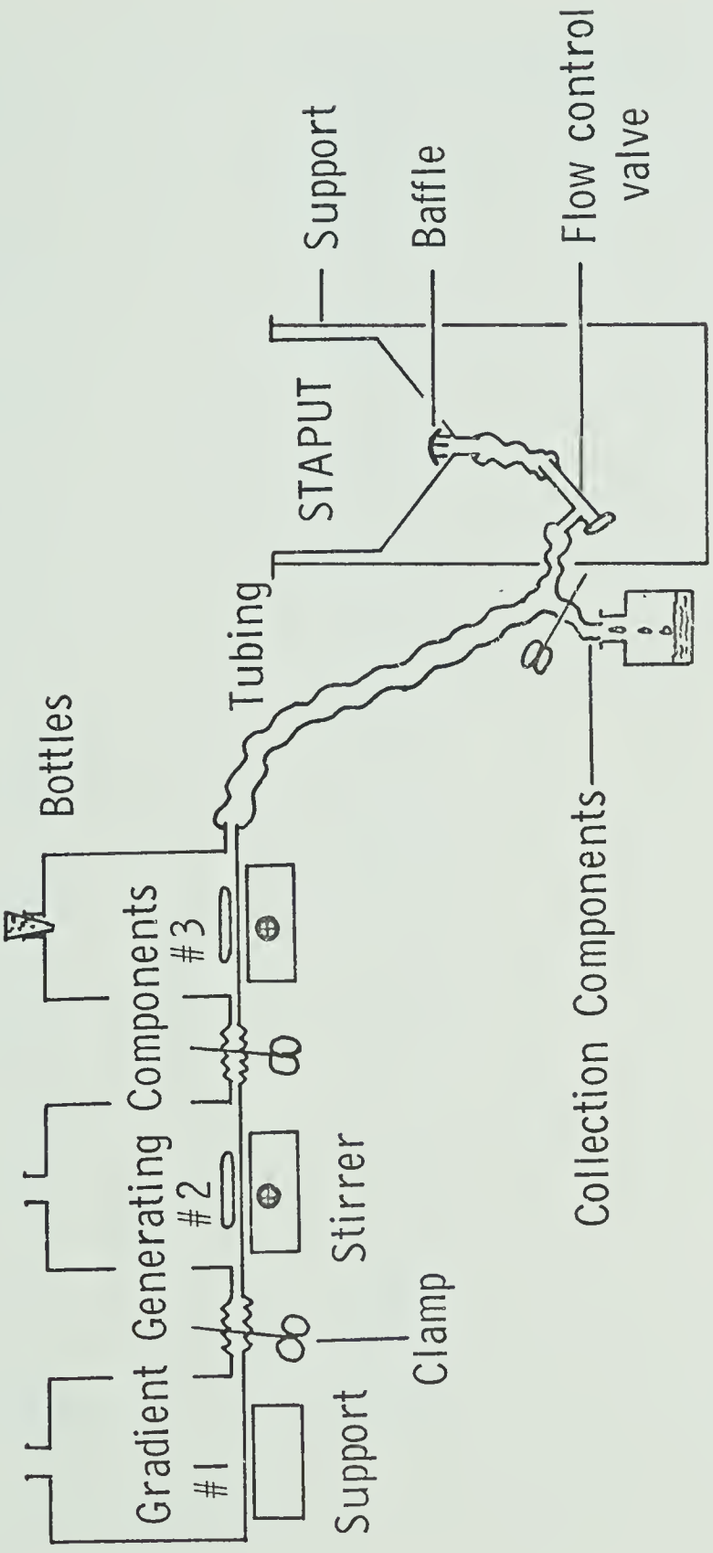

Bottles
Gradient Generating Components
#1
#2
#3
Support
Clamp
Stirrer
Tubing
STAPUT
Support
Baffle
Flow control valve
Collection Components

Figure 5 Karotype of L5178Y

[41 acrocentric chromosomes (s)]

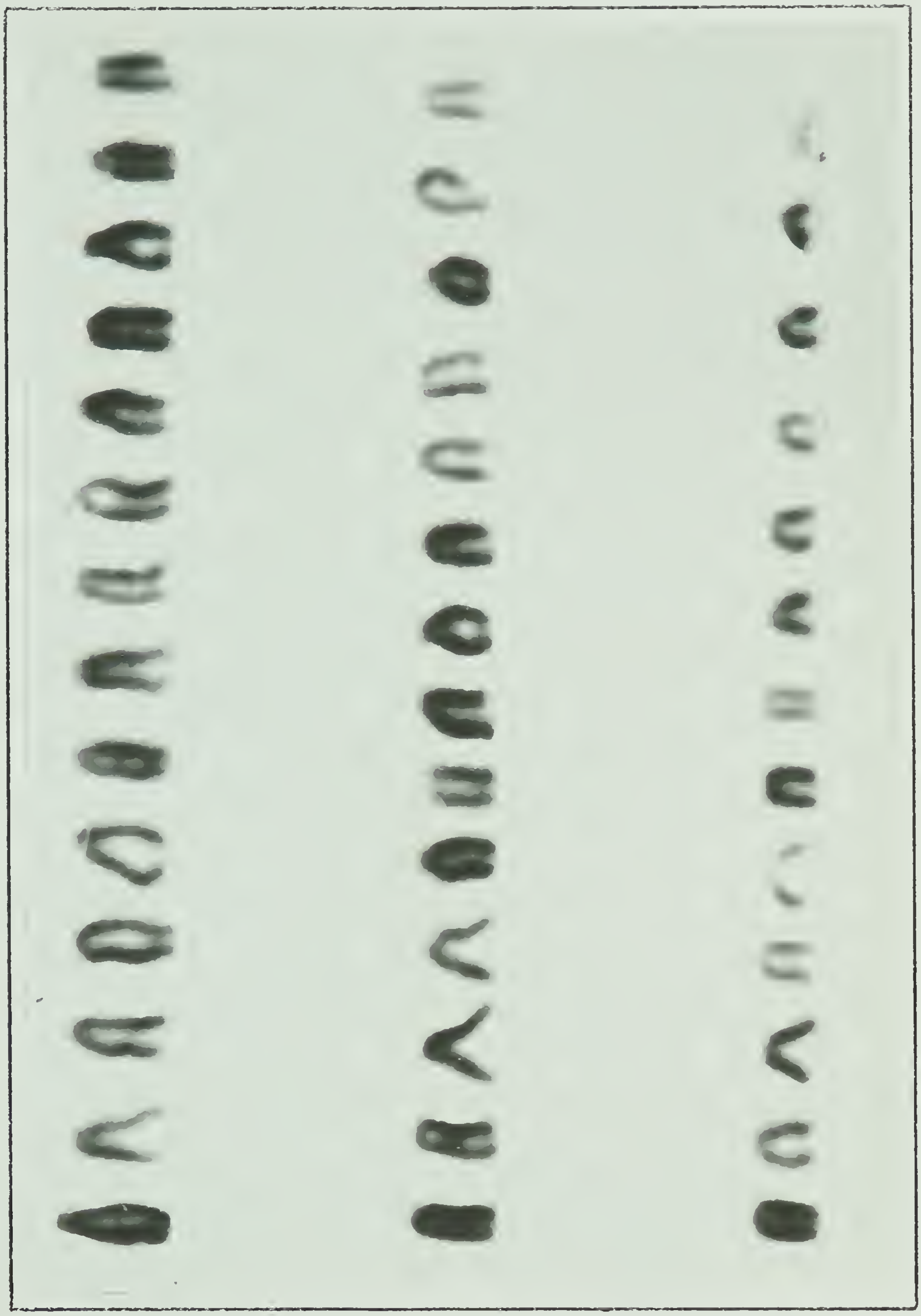


Figure 6 Velocity Sedimentation Profile Expt. 27

30.0×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 65 minutes, 43 fractions were collected.

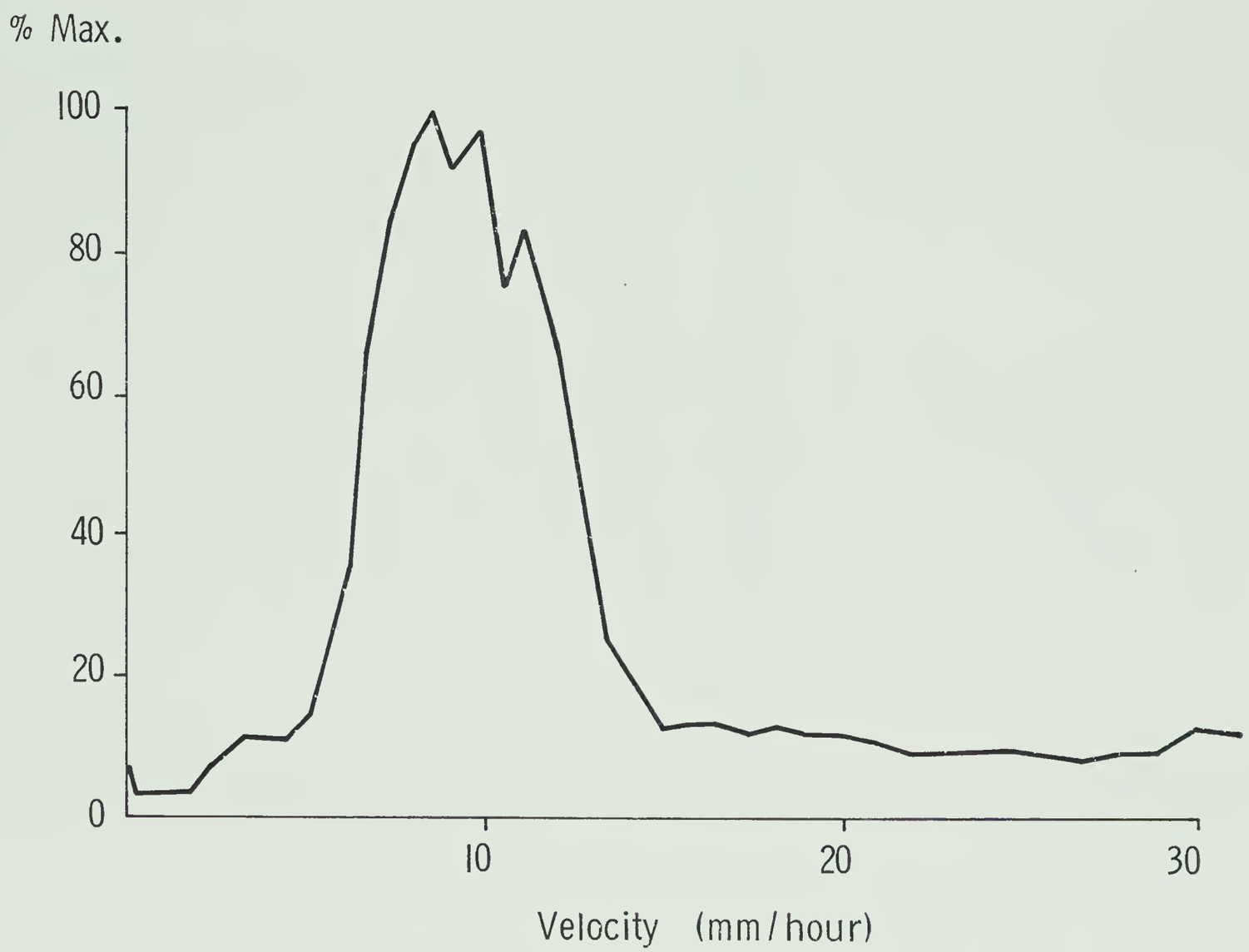

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 7 Velocity Sedimentation Profile Expt. 30

9.6×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 68 minutes, 45 fractions were collected.

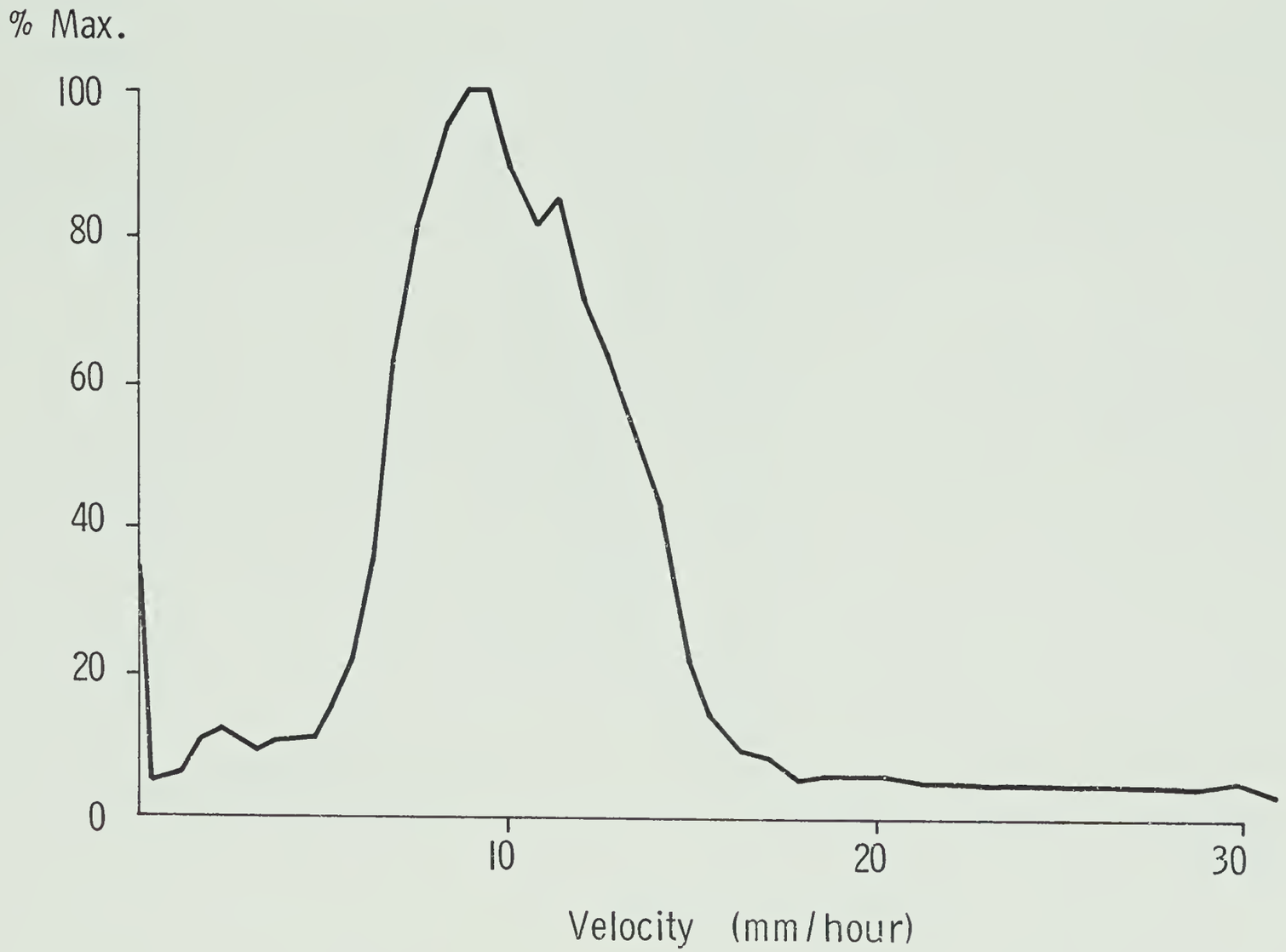

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 8 Velocity Sedimentation Profile Expt. 32

22.0×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 108 minutes, 43 fractions were collected.

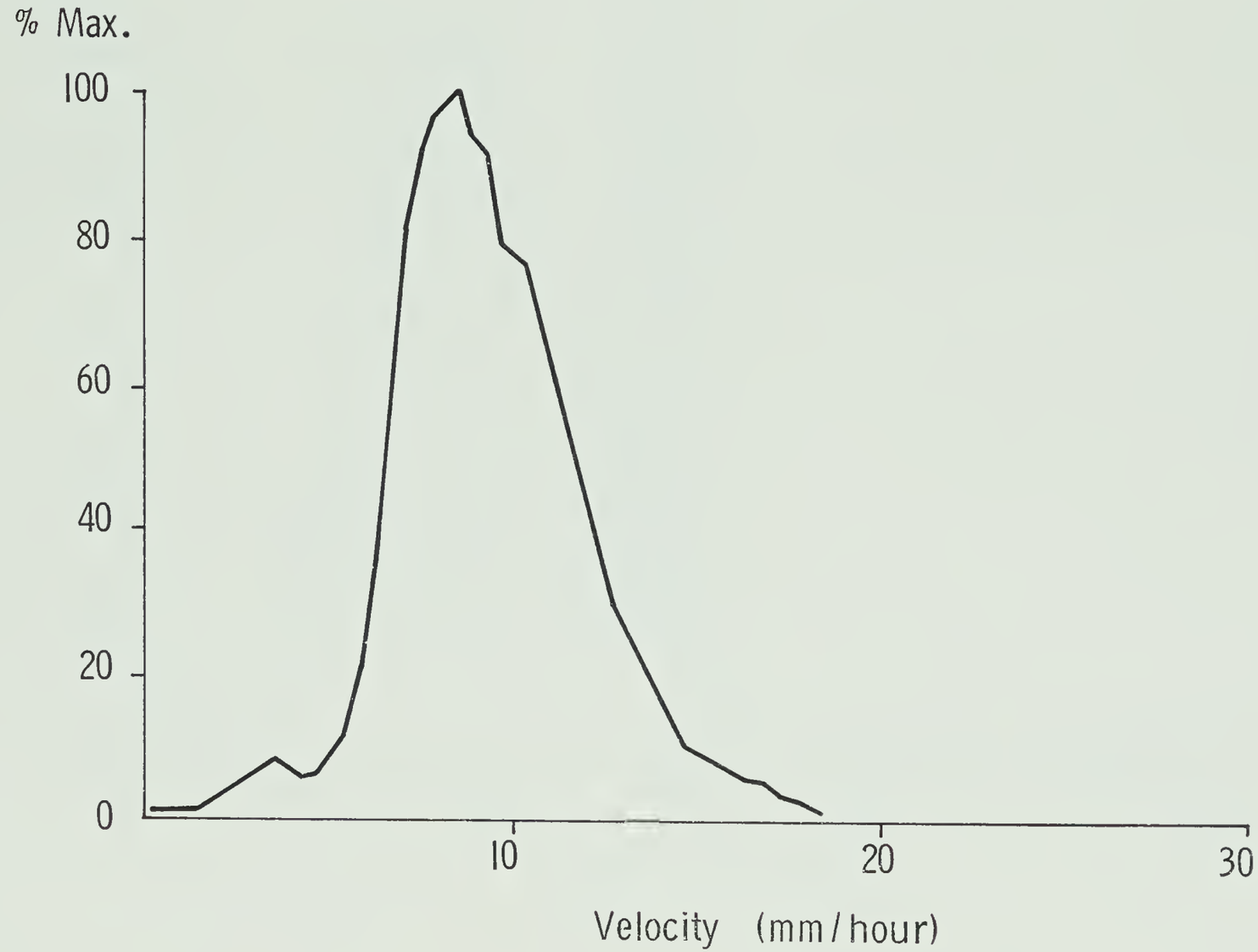

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 9 Composite Sedimentation Profile of 5 day *in vivo* tumour, numerically derived from Expts. 27, 30, 32

An average of 20.5×10^7 cells was loaded into the STAPUT. After a mean Standing time (T_s) of 80 minutes, an average of 44 fractions was collected.

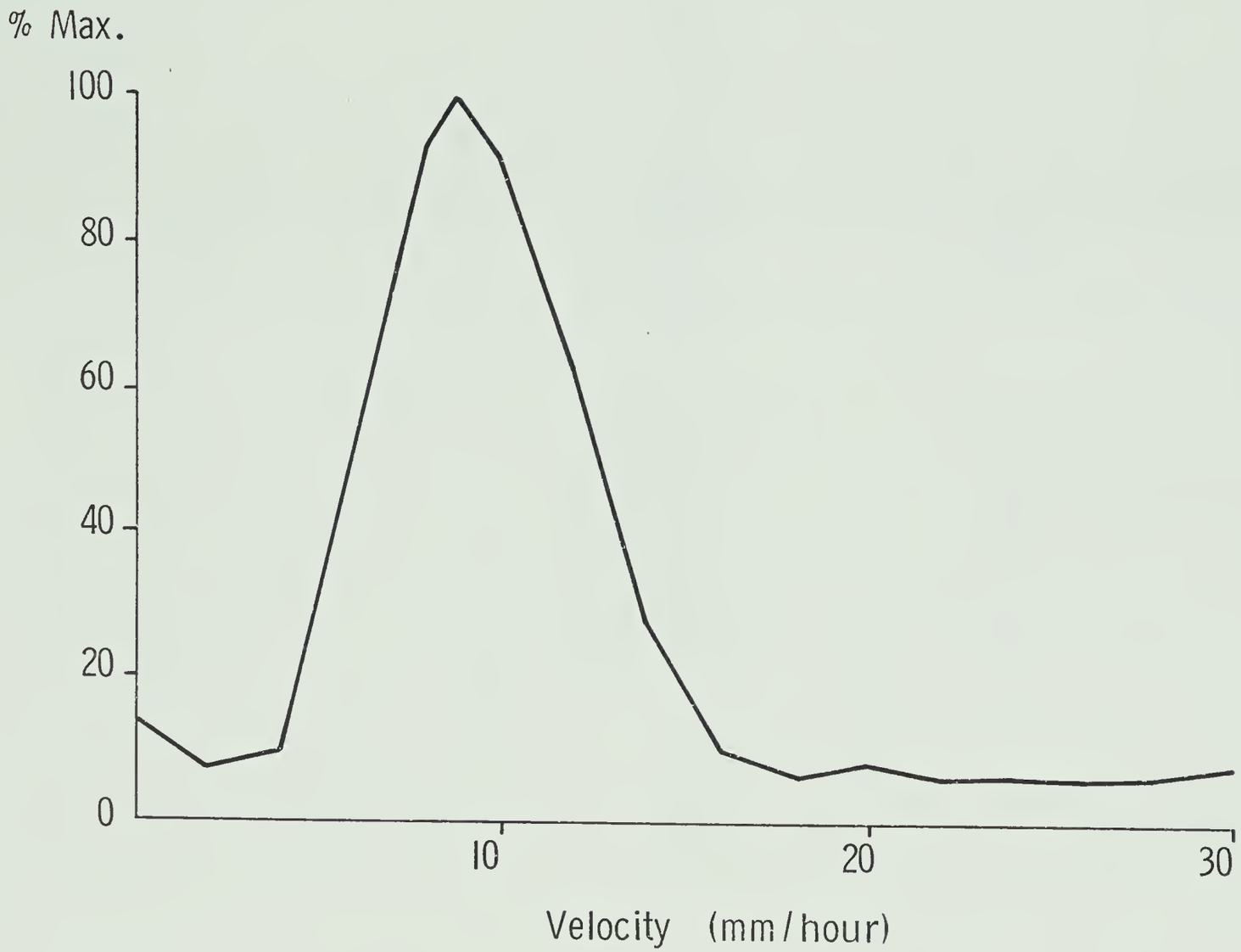

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 10 Vertical display of sedimentation profiles of *in vivo* cultured tumours
Expts. 27, 30, 32

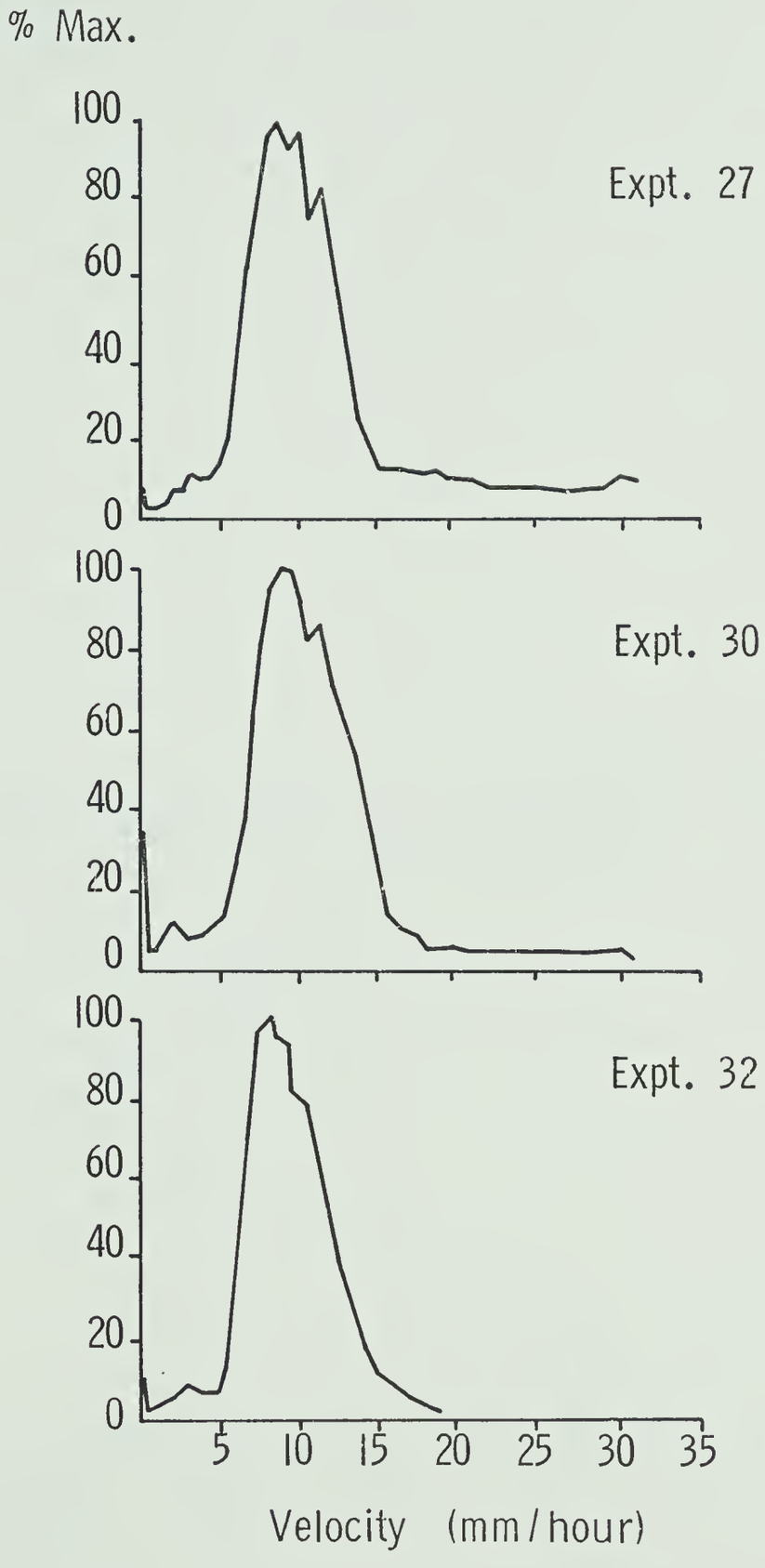

% Max.
100
80
60
40
20
0
Expt. 27
Expt. 30
Expt. 32
5
10
15
20
25
30
35
Velocity (mm/hour)

Figure II 'Streaming' in a sedimentation profile
Expt. 28

10.8×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 77 minutes, 42 fraction were collected.


% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 12 Effect of 'Streaming', in comparison to normal profile of 5 day *in vivo* tumour

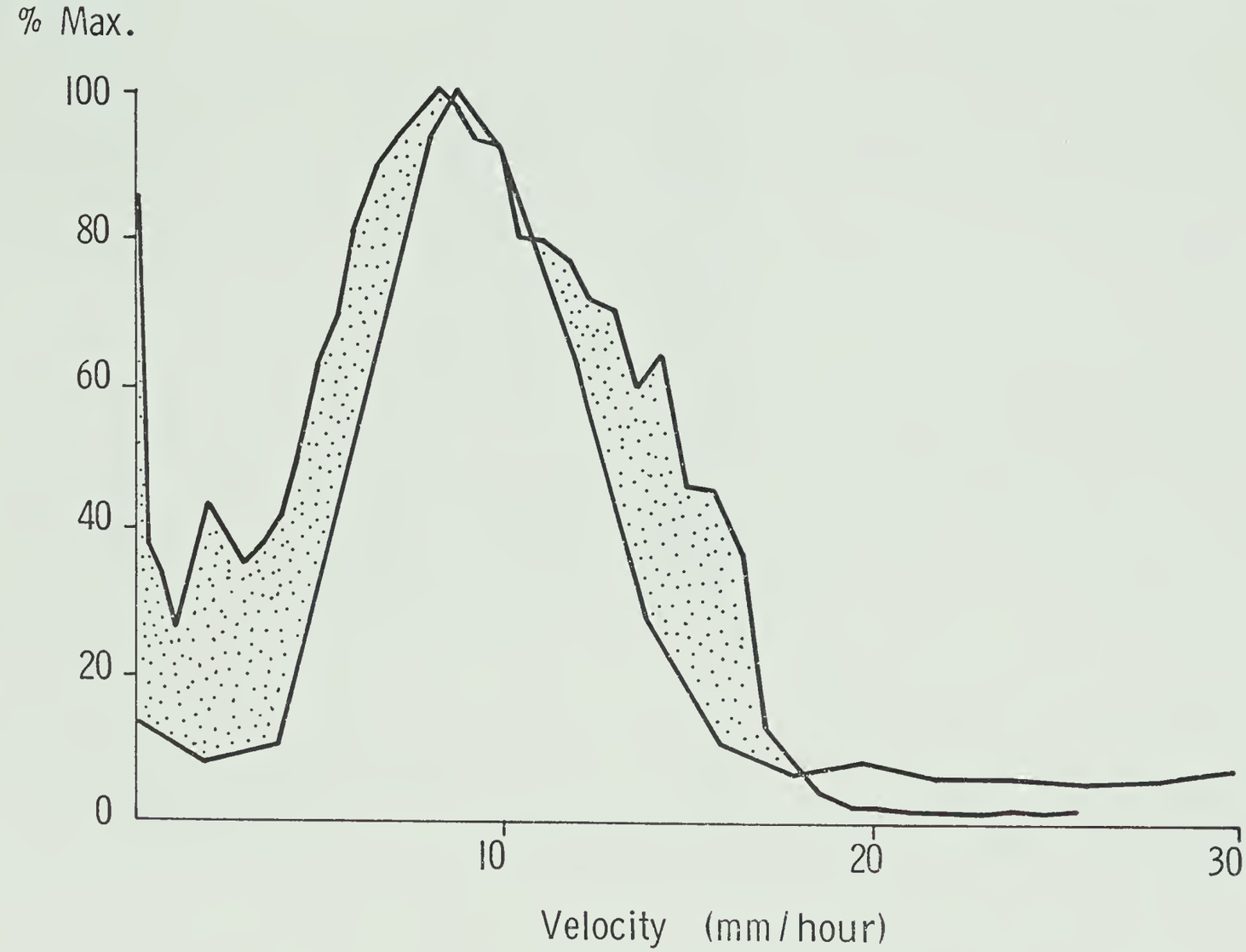

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm / hour)

Figure 13 'Giant' and non-viable cells in a sedimentation population Expt. 29

22×10^7 cells were loaded into the STAPUT. Of these, 6×10^6 cells were 'giant' cells and 18×10^6 cells were non-viable. After a Standing time (T_s) of 61 minutes, 44 fractions were collected.

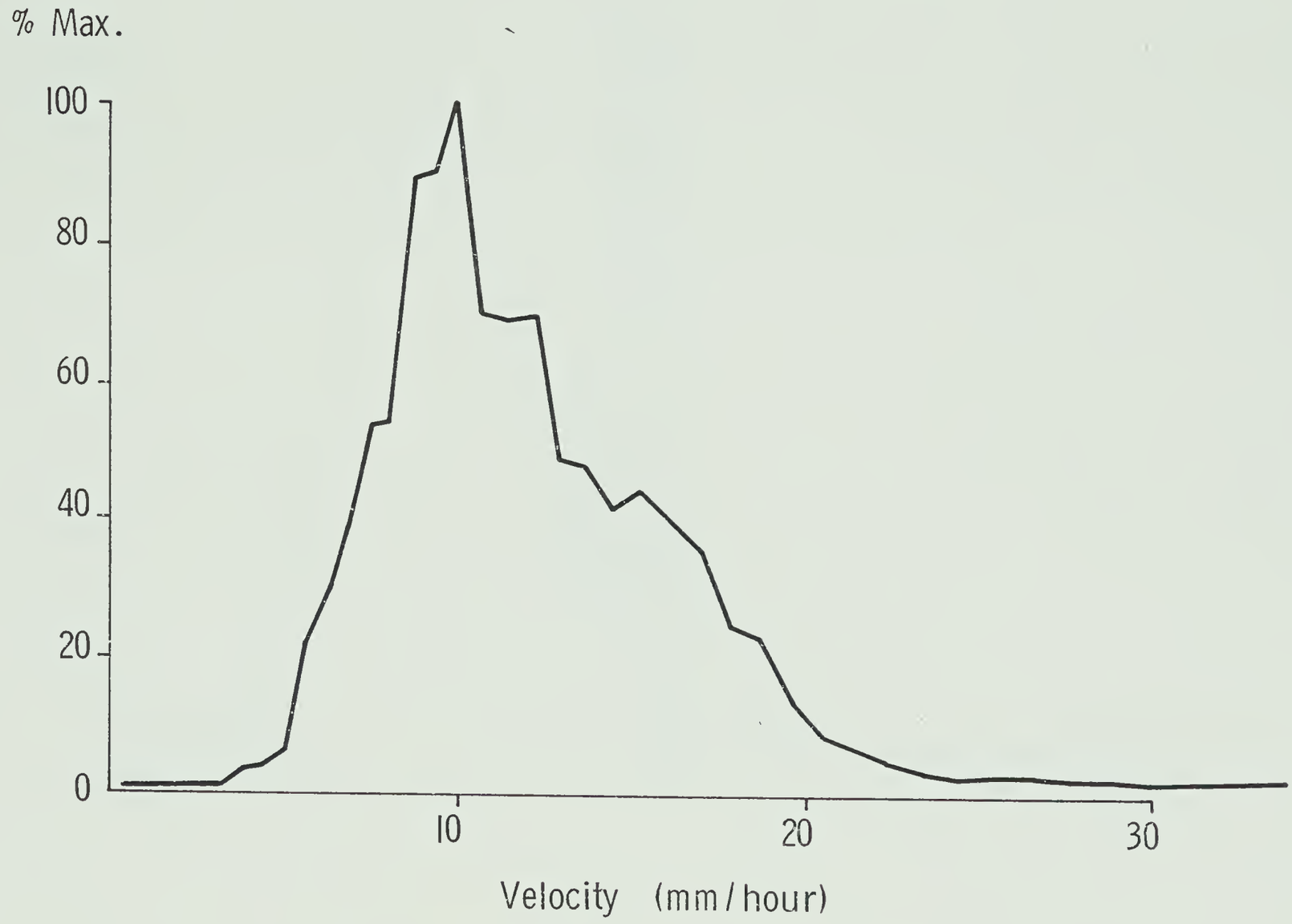

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 14 Effect of 'giant' and non-viable cells, in comparison to normal profile of 5 day *in vivo* tumour

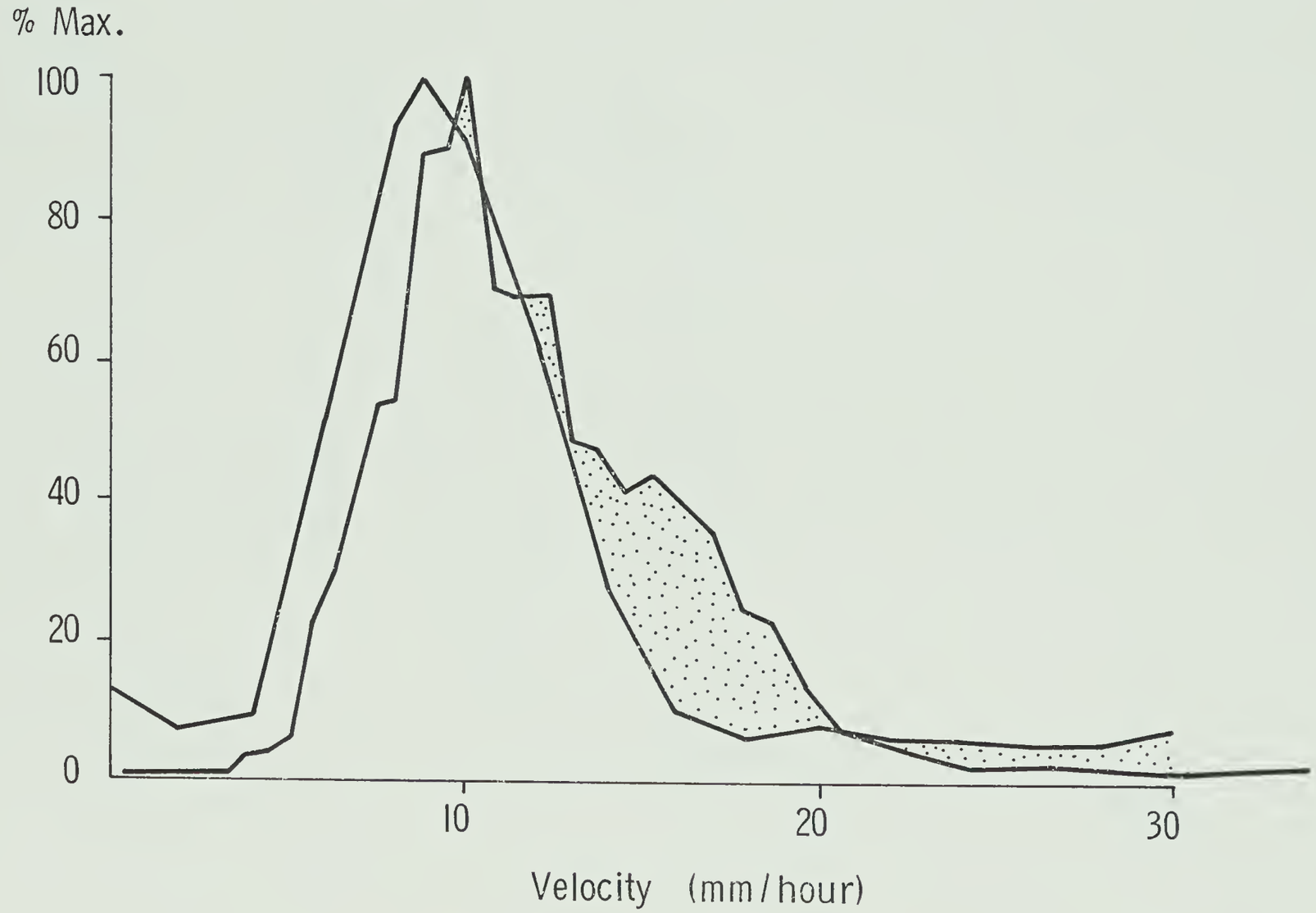

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 15 Sedimentation Profile Expt. 9

15×10^7 cells were loaded into the STAPUT.

After a Standing time (T_s) of 91 minutes,

43 fractions were collected.

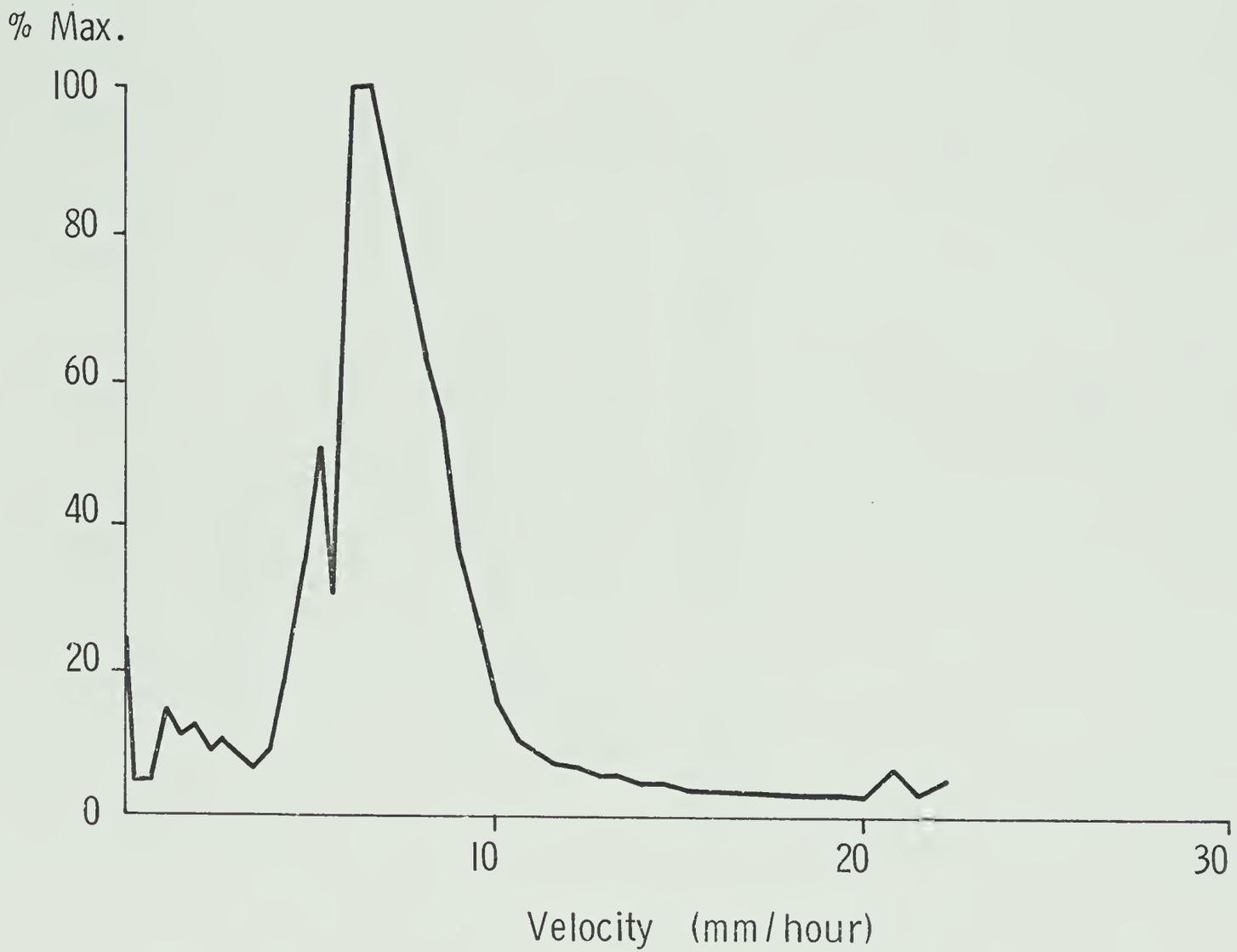

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 16 Sedimentation Profile Expt. 7

23×10^7 cells were loaded into the STAPUT.

After a Standing time (T_s) of 88 minutes,

42 fractions were collected.

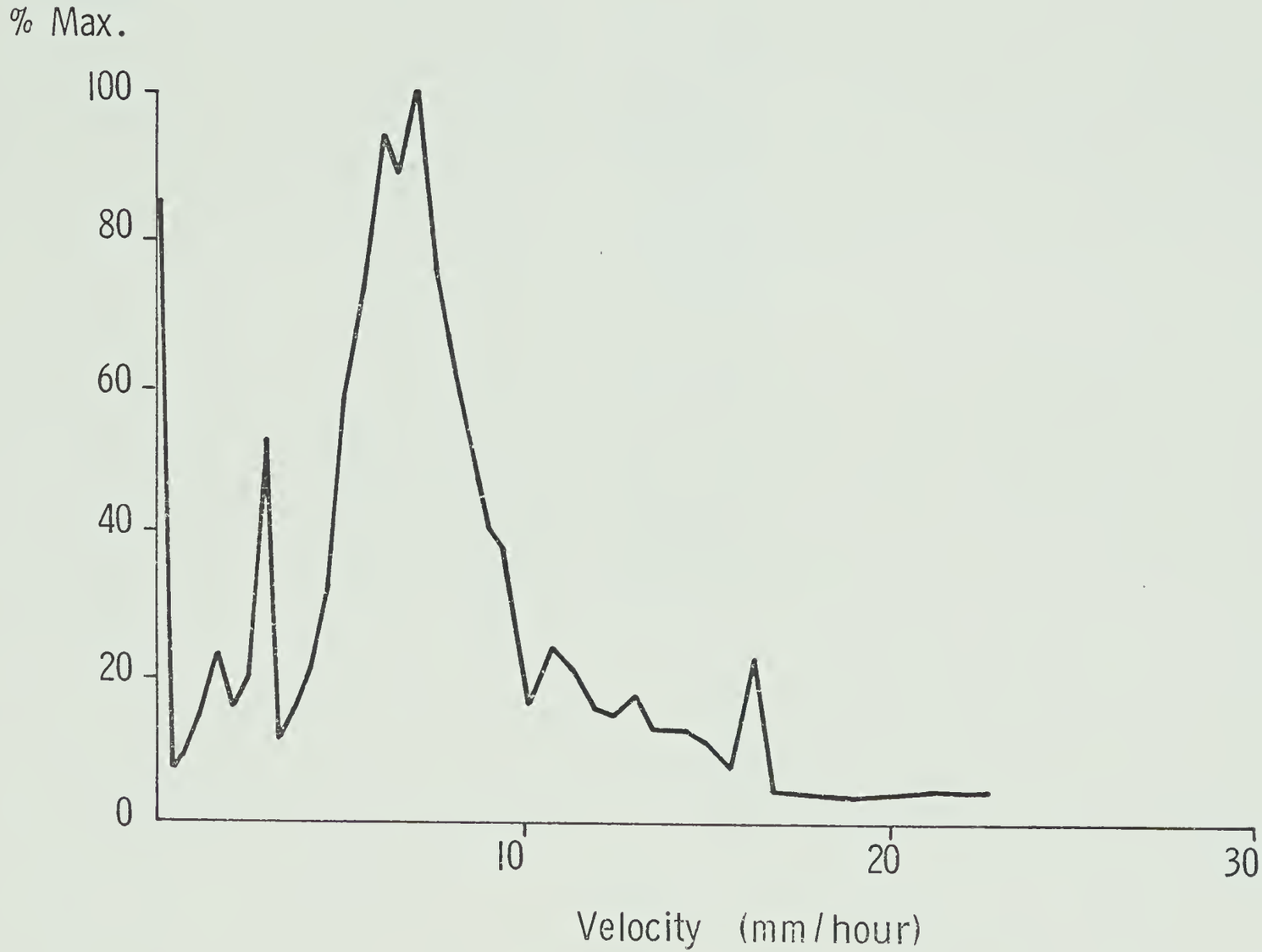

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 17 Composite sedimentation profile, of 5 day *in vivo*, 1-2 day *in vitro* tumour, numerically derived from Expts. 9 & 7

An average of 19×10^7 cells was loaded into the STAPUT. After a mean Standing time (T_s) of 89 minutes, an average of 42.5 fractions was collected.

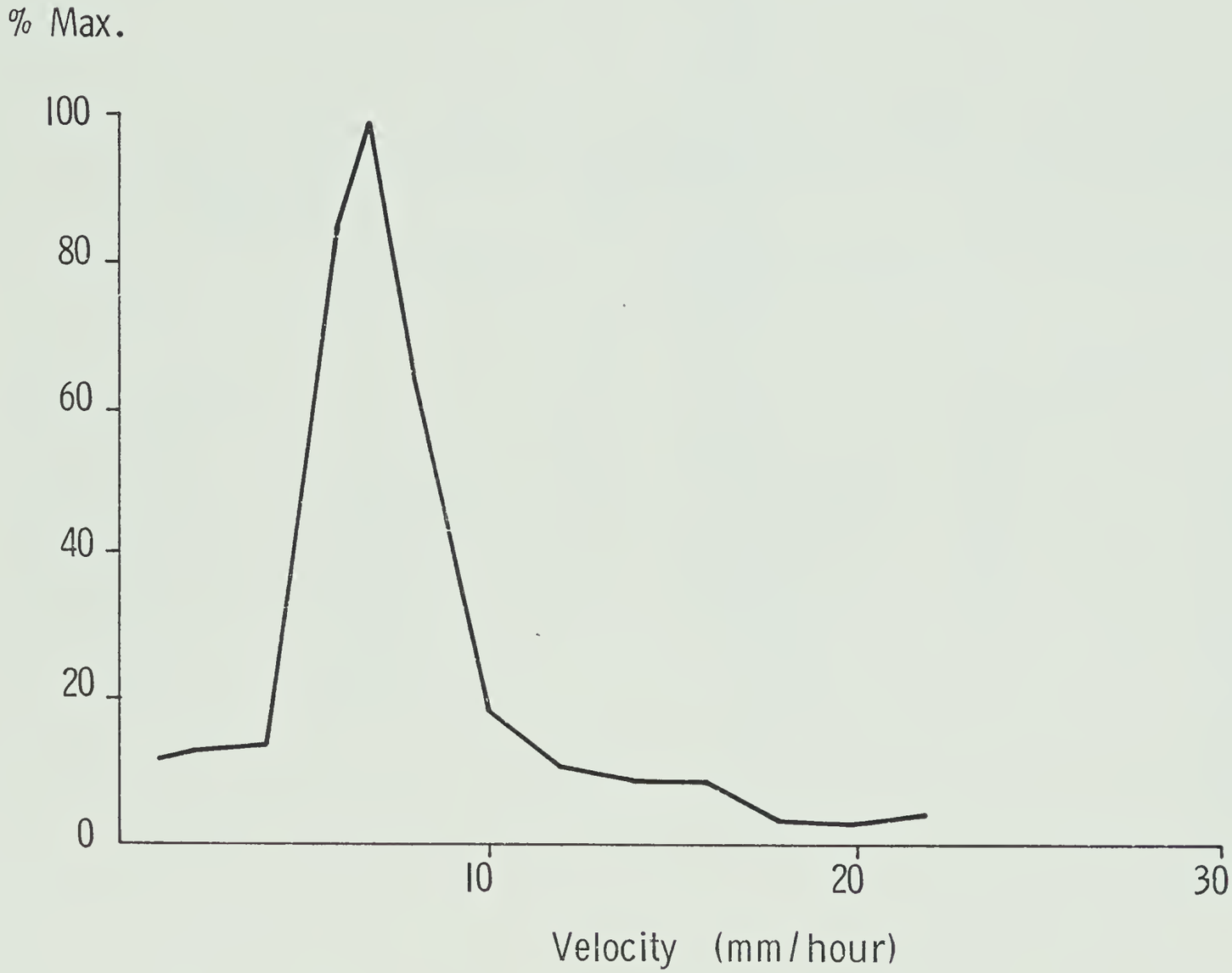

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 18 Vertical display of sedimentation profiles of tumours with varying *in vitro* time periods

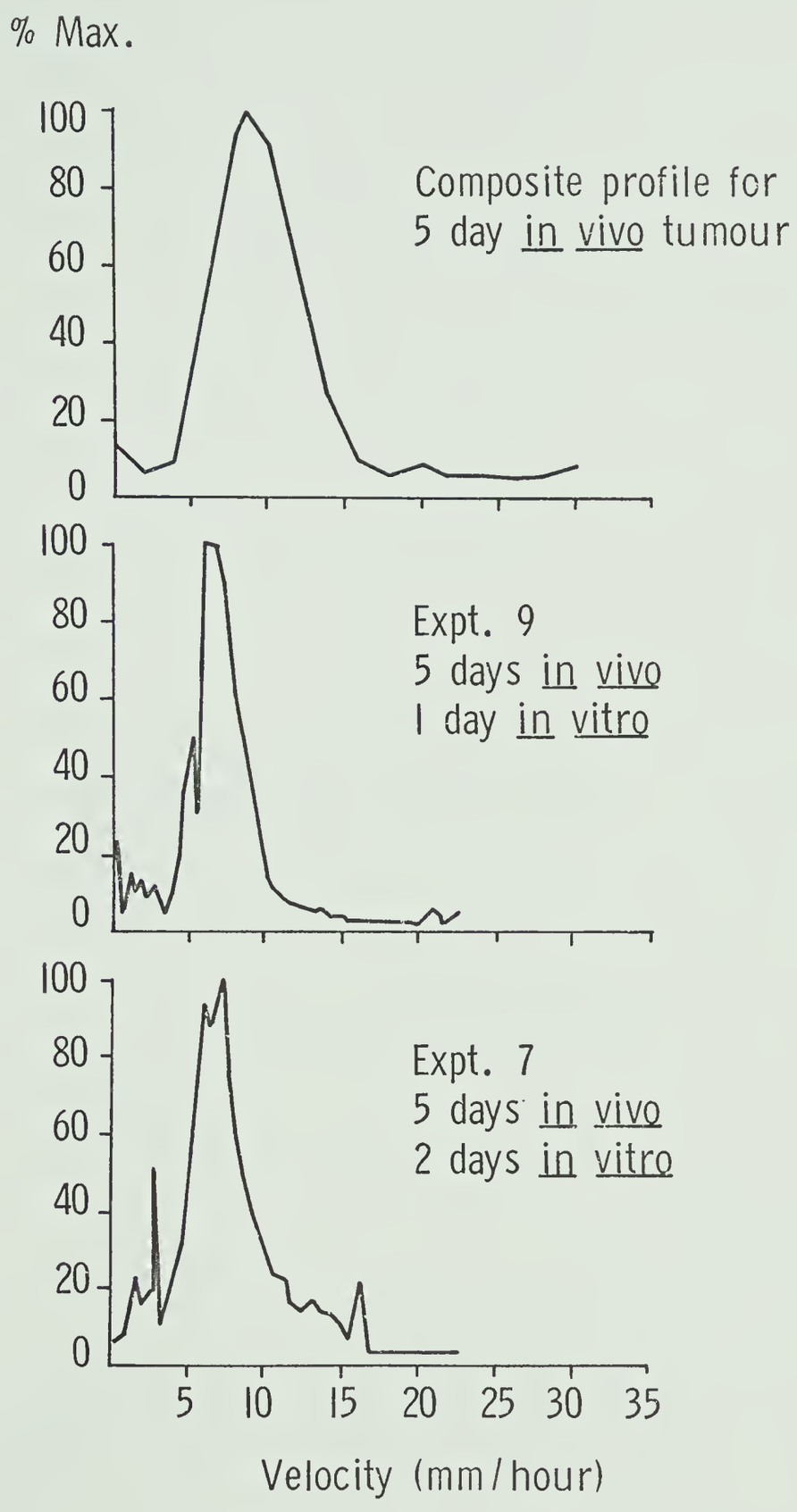

% Max.
100
80
60
40
20
0
Composite profile for
5 day in vivo tumour
Expt. 9
5 days in vivo
1 day in vitro
Expt. 7
5 days in vivo
2 days in vitro
5
10
15
20
25
30
35
Velocity (mm/hour)

Figure 19 Sedimentation profile Expt. 24

5.0×10^7 cells were loaded into the STAPUT.

After a Standing time (T_s) of 91 minutes,

42 fractions were collected.

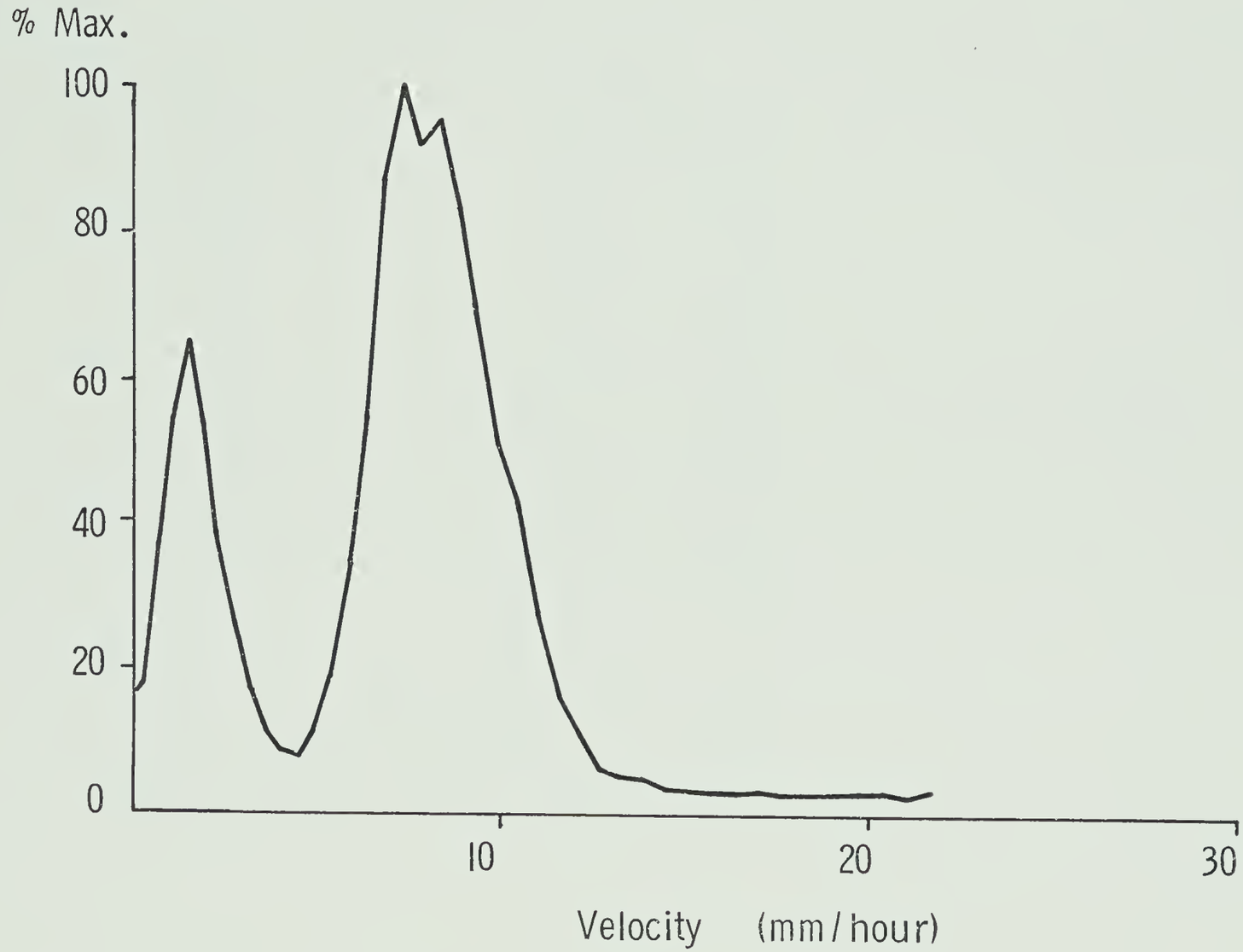

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 20 Sedimentation profile Expt. 25

30.0×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 96 minutes, 44 fractions were collected.

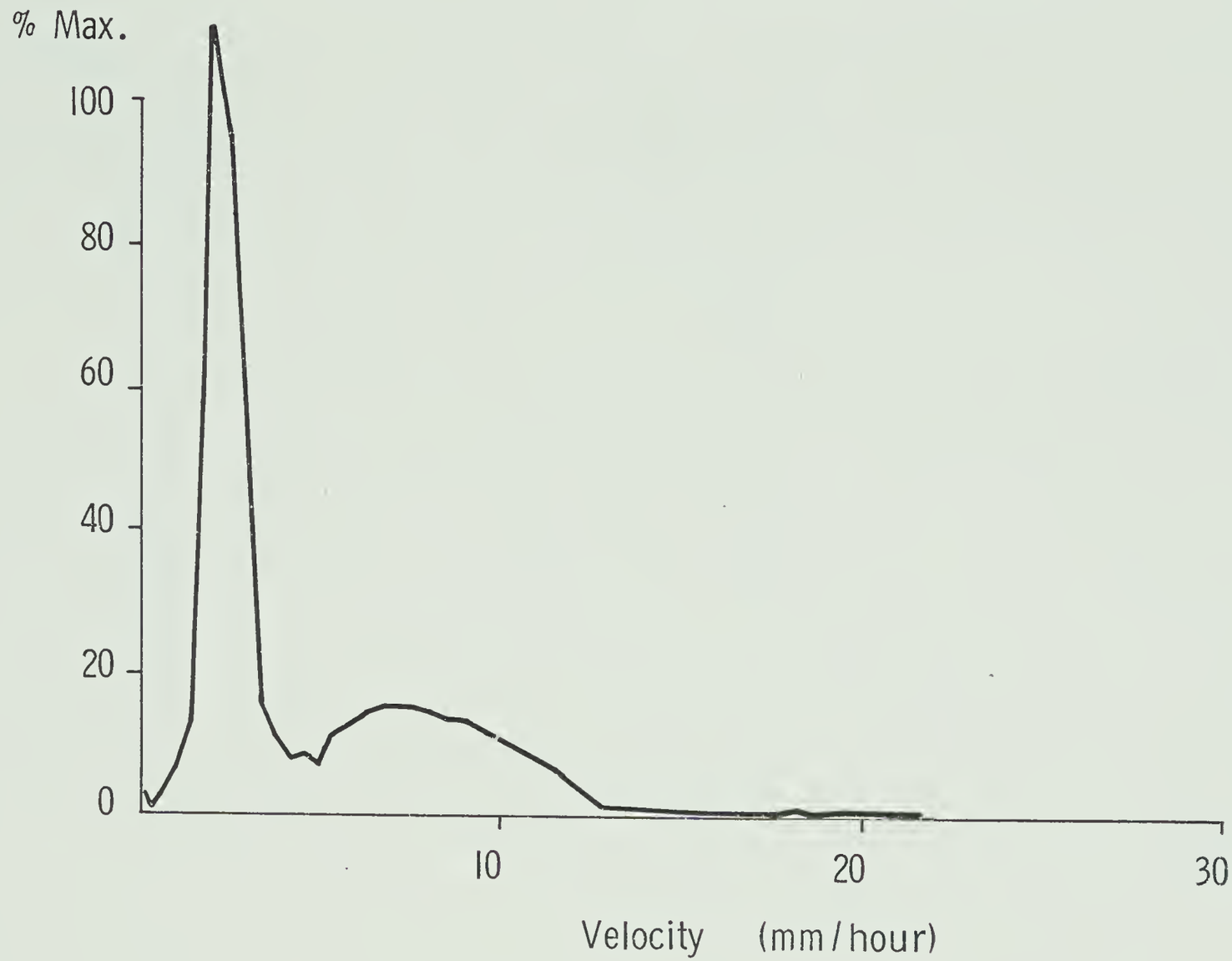

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 21 Sedimentation Profile Expt. 21

22.5×10^7 cells were loaded into the STAPUT.

After a Standing time (T_s) of 107 minutes,

39 fractions were collected.

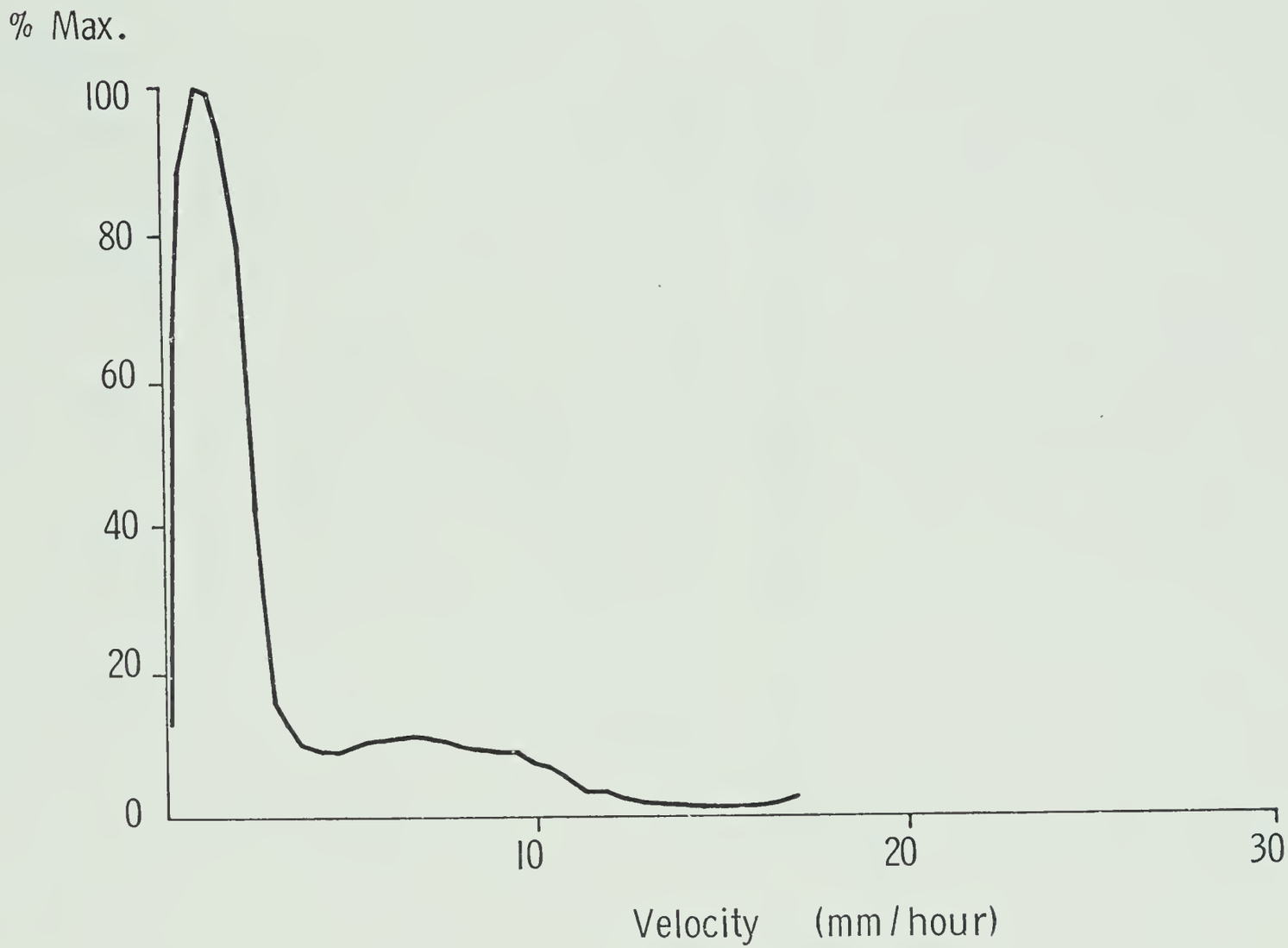

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 22 Sedimentation Profile Expt. 22

15.0×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 92 minutes, 42 fractions were collected.


% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 23 Sedimentation Profile Expt. 23

50.0×10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 97 minutes, 44 fractions were collected.

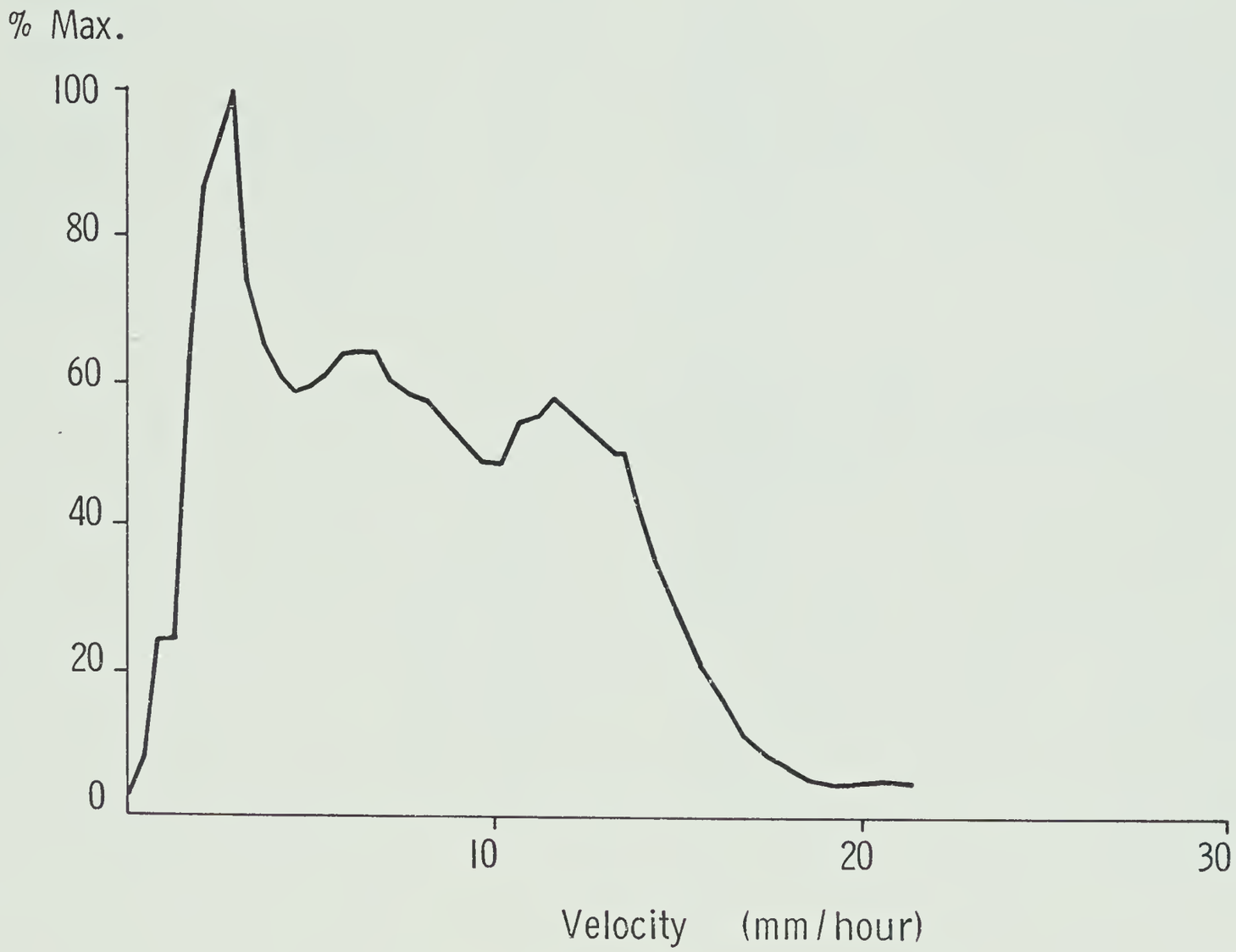

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 24 Vertical display of sedimentation profiles of tumours with varying *in vitro* time periods (Expt. 21-25)

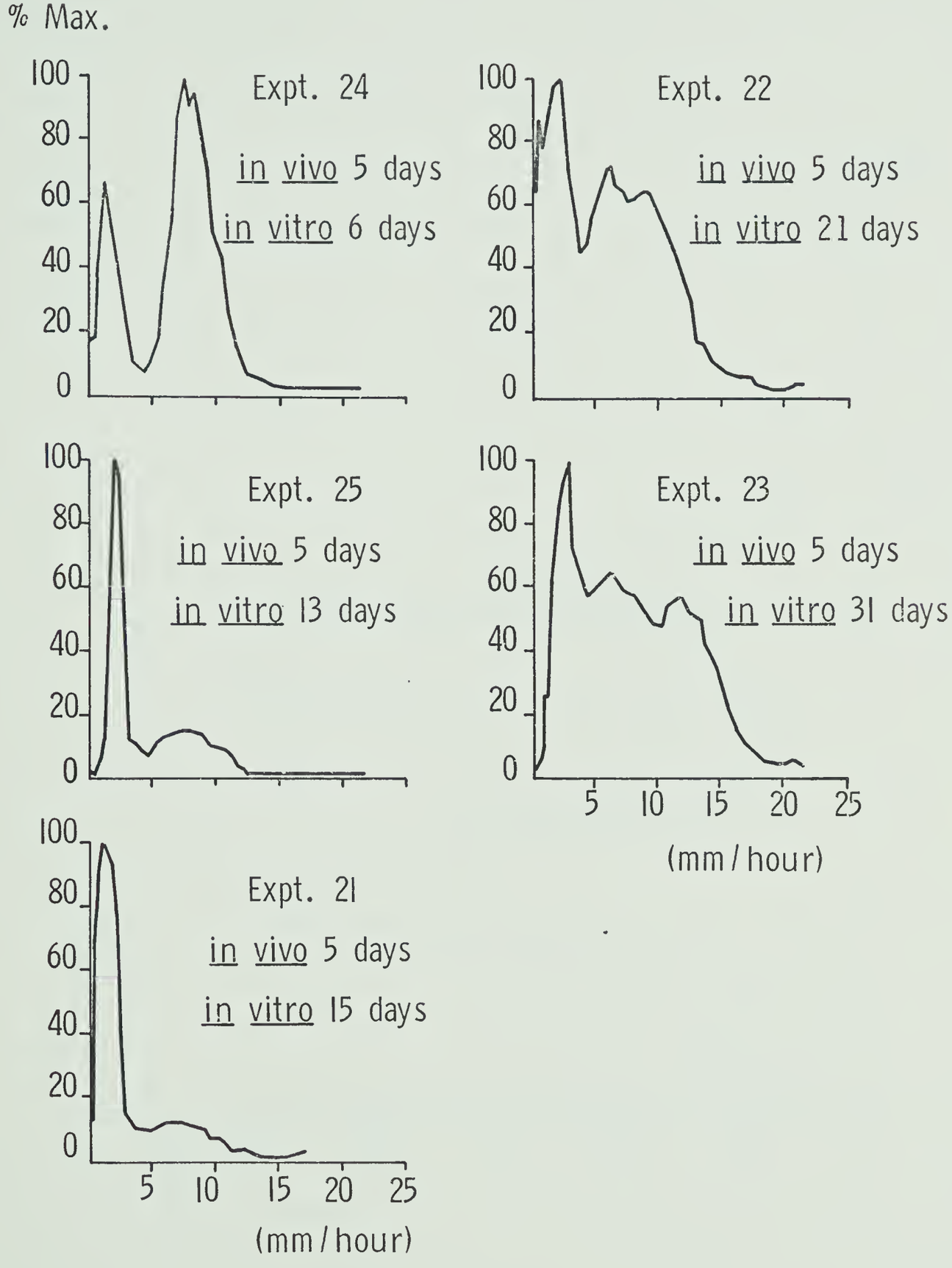

% Max.
100
80
60
40
20
0
Expt. 24
in vivo 5 days
in vitro 6 days
Expt. 22
in vivo 5 days
in vitro 21 days
Expt. 25
in vivo 5 days
in vitro 13 days
Expt. 23
in vivo 5 days
in vitro 31 days
Expt. 21
in vivo 5 days
in vitro 15 days
5
10
15
20
25
(mm/hour)

Figure 25 Sedimentation Profile Expt. 5

24×10^7 cells were loaded into the STAPUT.

After a Standing time (T_s) of 101 minutes,

43 fractions were collected.

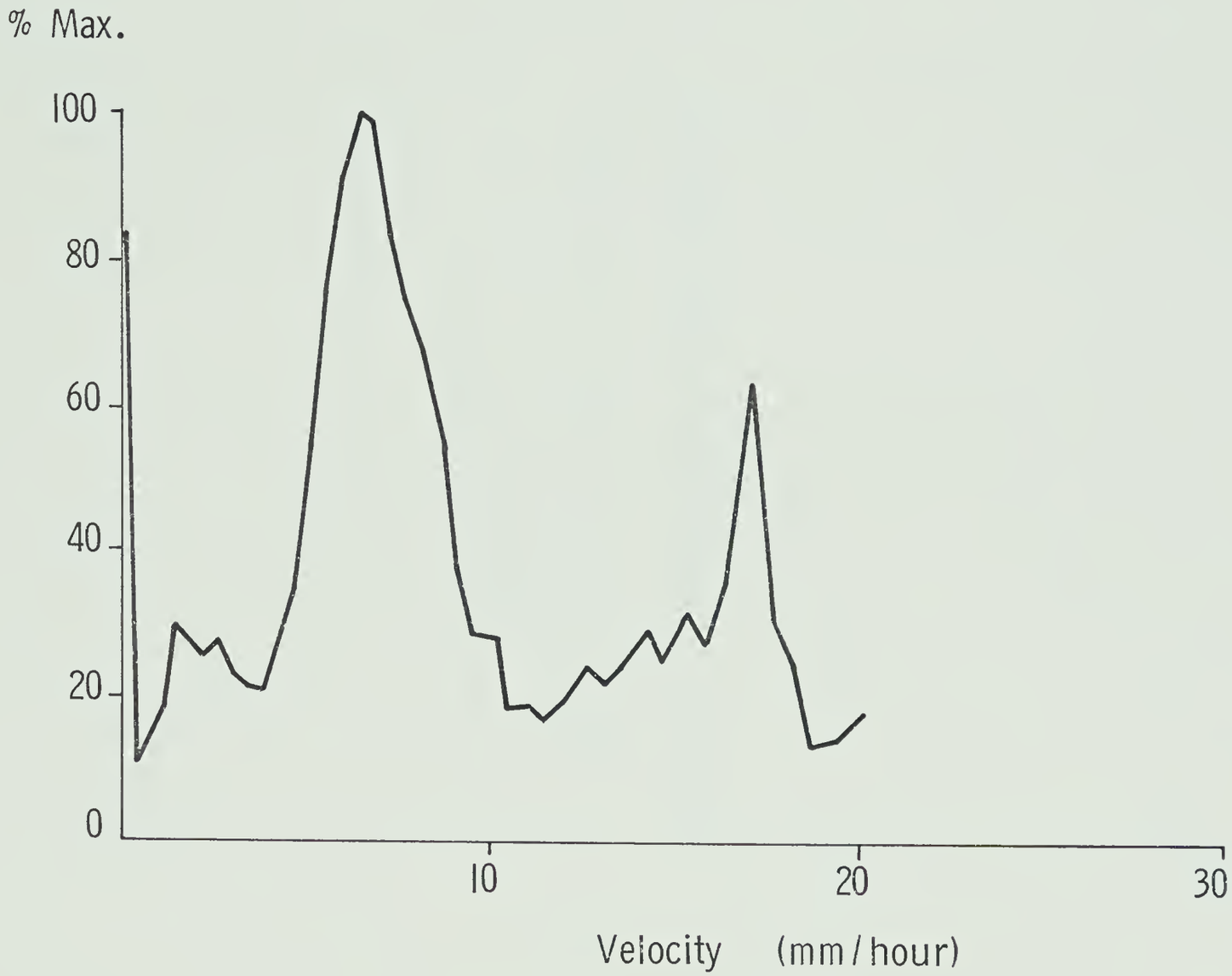

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 26 Sedimentation Profile Expt. 12

28×10^6 cells were loaded into the STAPUT. After a Standing time (T_S) of 103 minutes, 42 fractions were collected.

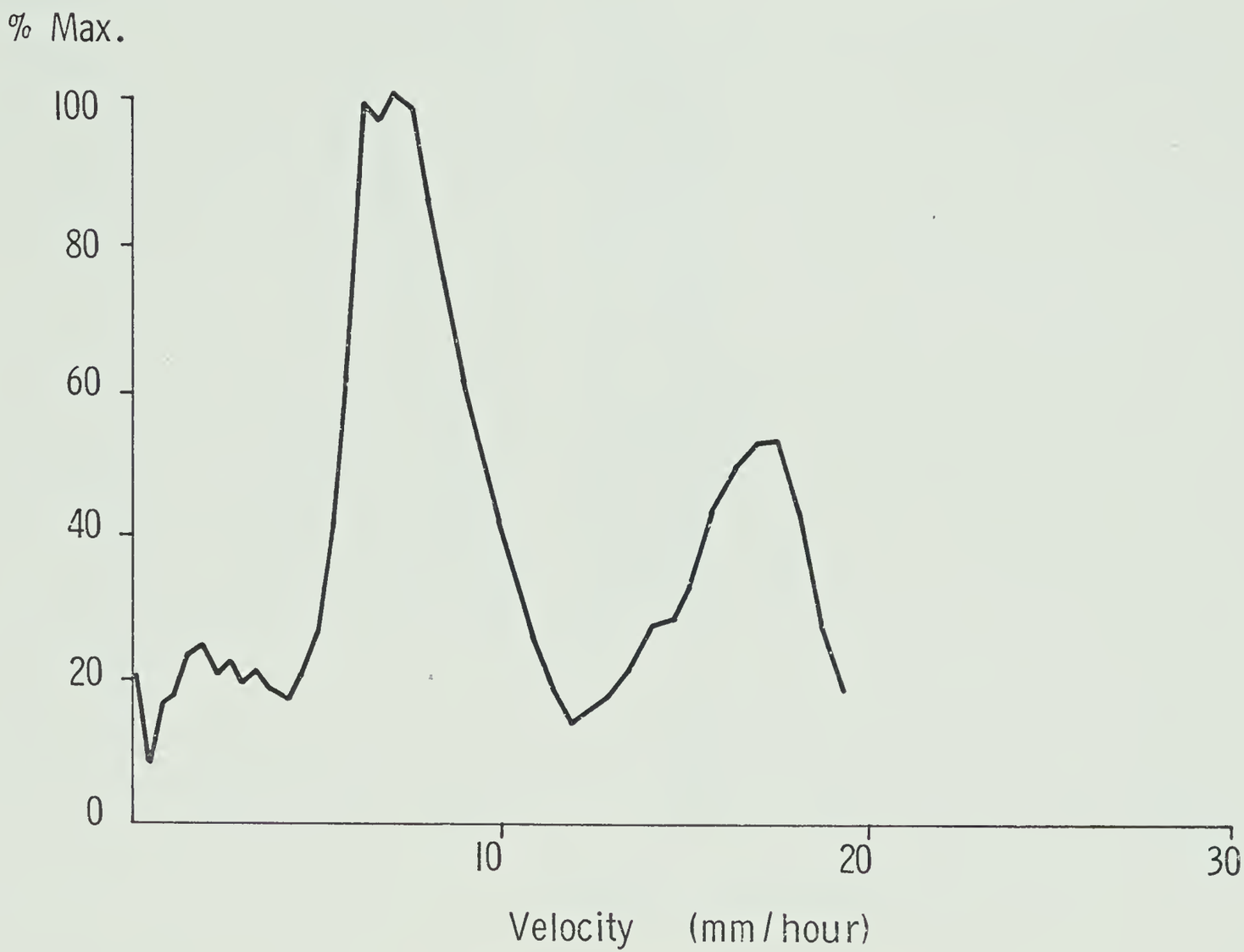

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 27 Composite sedimentation profile of 7 day *in vivo*, 5 day *in vitro* tumour, numerically derived from Expt. 5 & 12

An average of 26×10^7 cells was loaded into the STAPUT. After a mean Standing time (T_s) of 102 minutes, an average of 42.5 fractions was collected.

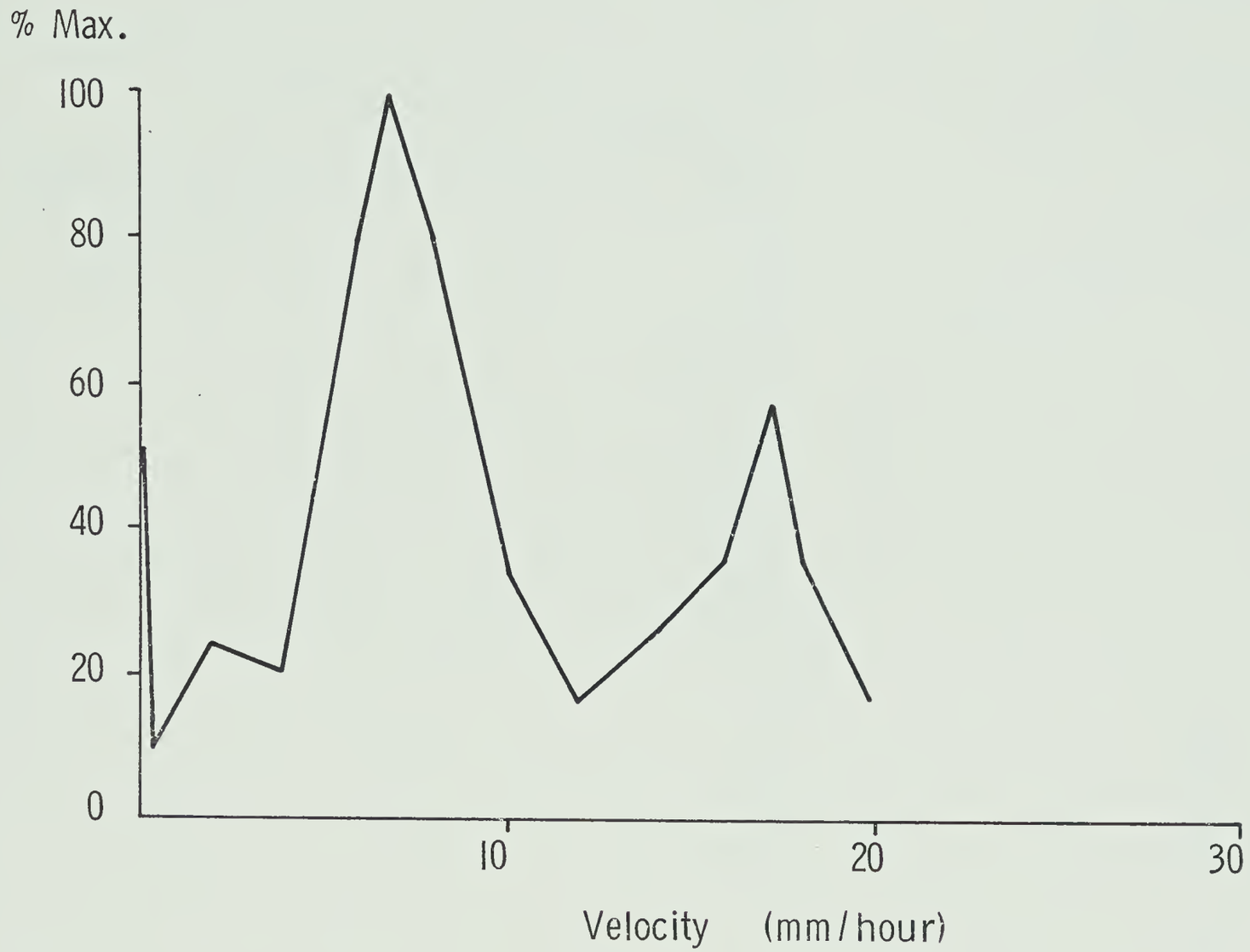

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 28 Sedimentation profile of fractions 23 - 29 after 8 days in vitro

In Experiment 6, 20 x 10^7 cells were loaded into the STAPUT. After a Standing time (T_s) of 92 minutes, 42 fractions were collected. Fractions 32-29 were cultured *in vitro* for eight days. The cells from these fractions (17 x 10^7) were then loaded into the STAPUT (Expt. 10). After a Standing time (T_s) of 92 minutes, 44 fractions were collected.


% Max.
100
80
60
40
20
0
Recultured fractions
Expt. 10
Expt. 6
100
80
60
40
20
0
Expt. 10
10
20
30
(mm / hour)

Figure 29 Proportion of 2s cells in separated fractions (5 day *in vivo* tumour)

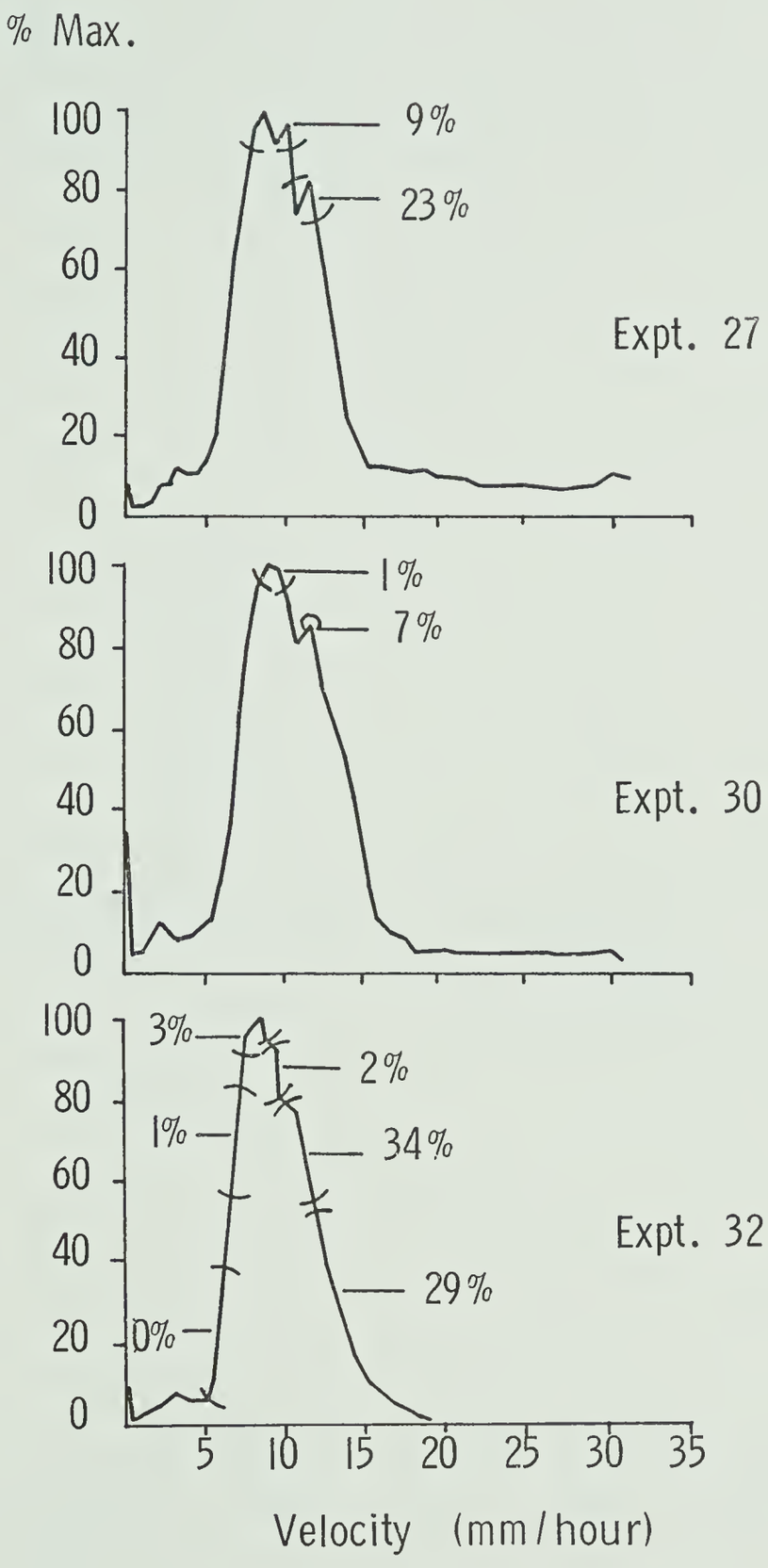

% Max.
100
80
60
40
20
0
9%
23%
Expt. 27
1%
7%
Expt. 30
3%
2%
1%
34%
29%
0%
Expt. 32
5
10
15
20
25
30
35
Velocity (mm/hour)

Figure 30 Distribution of 2s cells in 5 day *in vivo* tumour

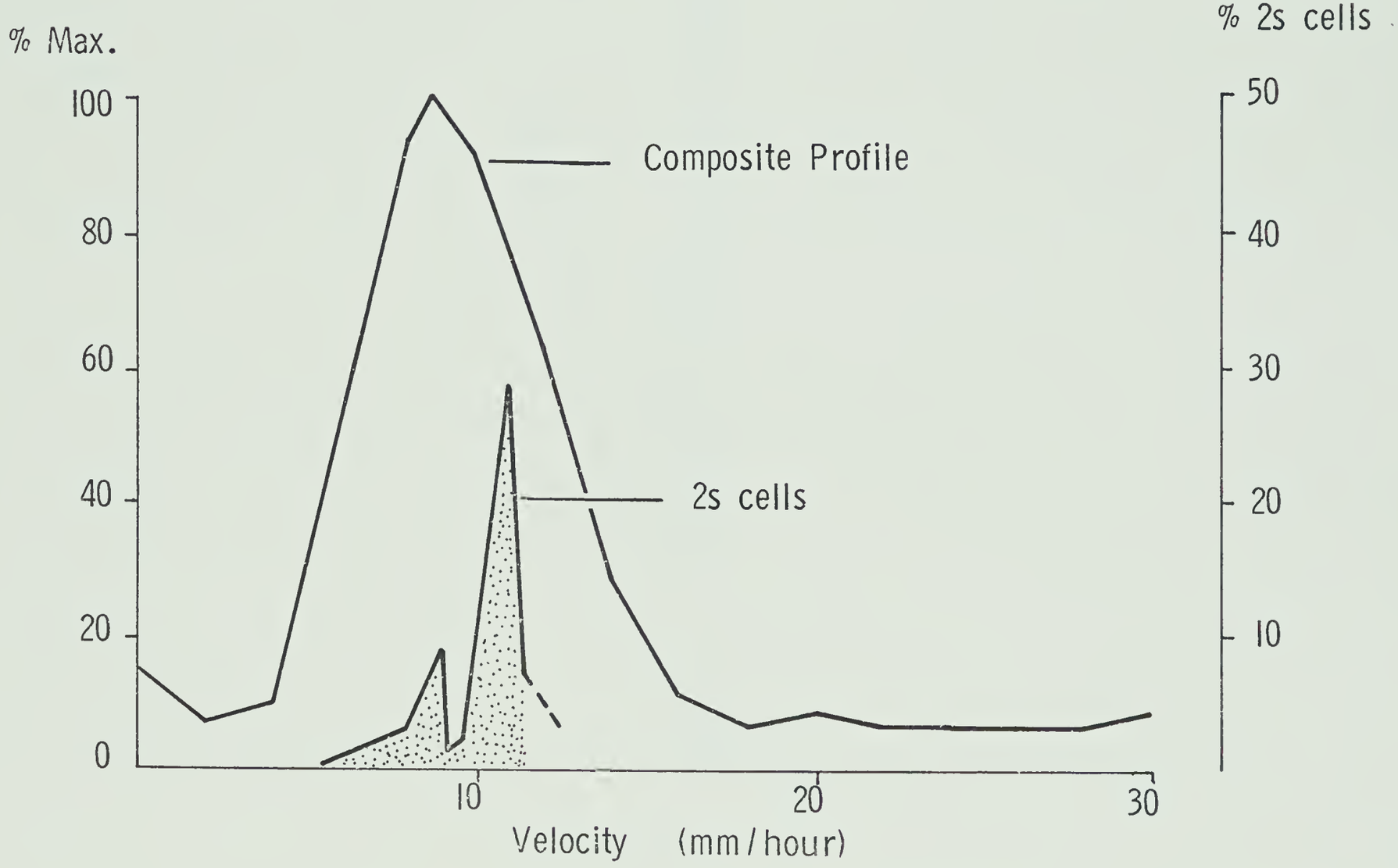

% Max.
% 2s cells
100
80
60
40
20
0
50
40
30
20
10
Composite Profile
2s cells
10
20
30
Velocity (mm/hour)

Figure 31 Distribution of measured cell diameters 5 day *in vivo* tumour

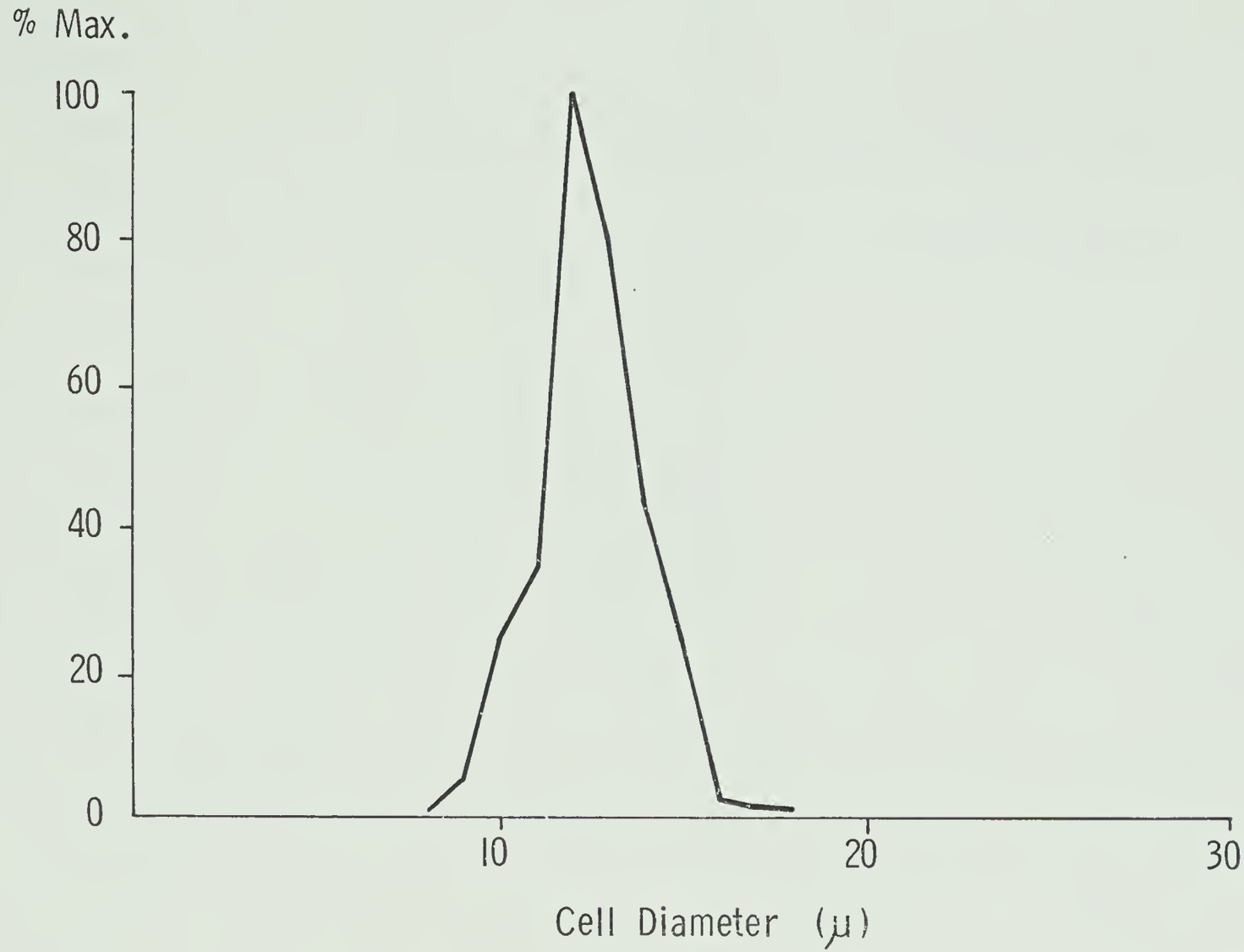

% Max.
100
80
60
40
20
0
10
20
30
Cell Diameter (μ)

Figure 32 Comparison of measured and calculated sedimentation profiles
(5 day in vivo tumour)

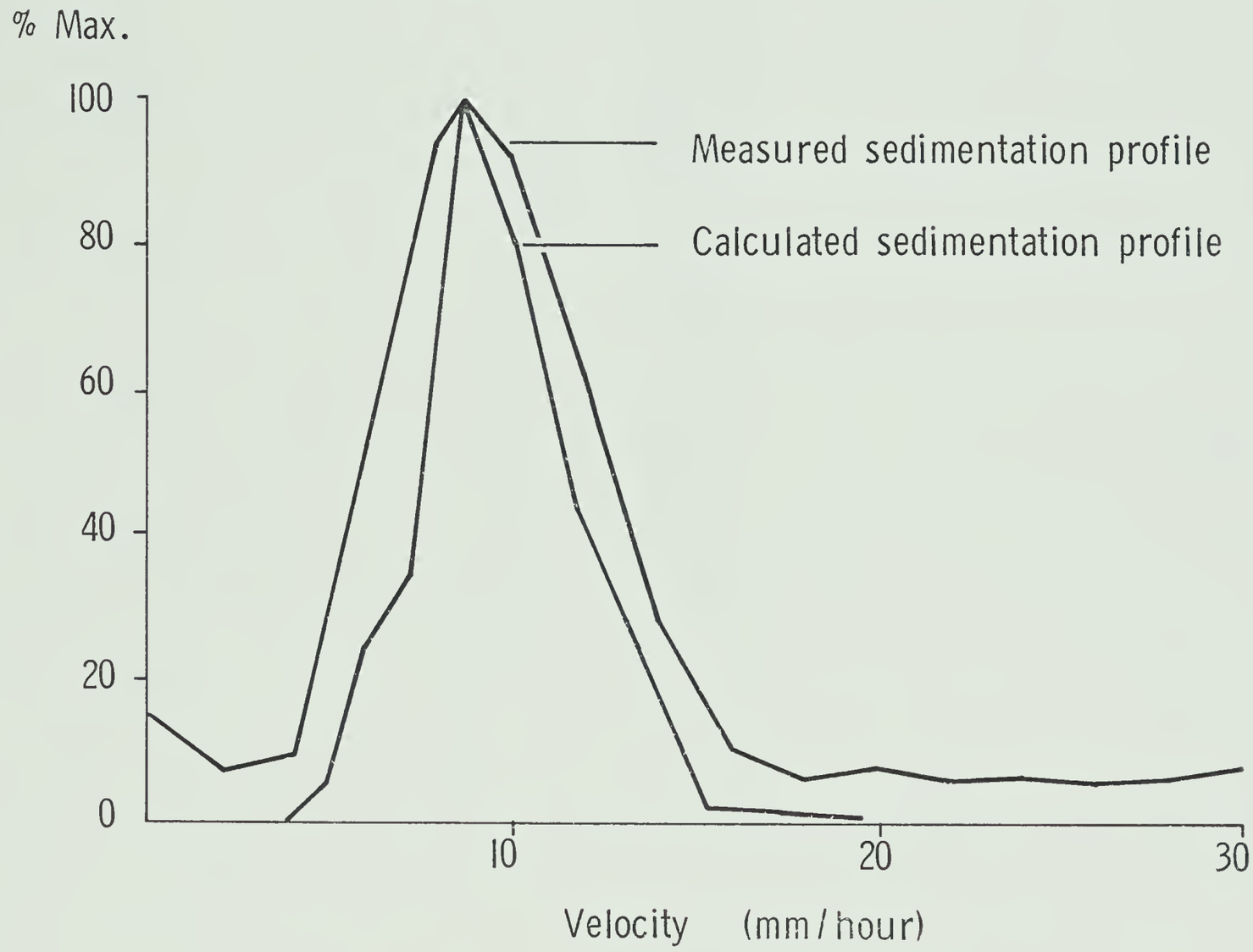

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)
Measured sedimentation profile
Calculated sedimentation profile

Figure 33 Distribution of measured cell diameters (5 day in vivo, 1-2 day in vitro tumour)

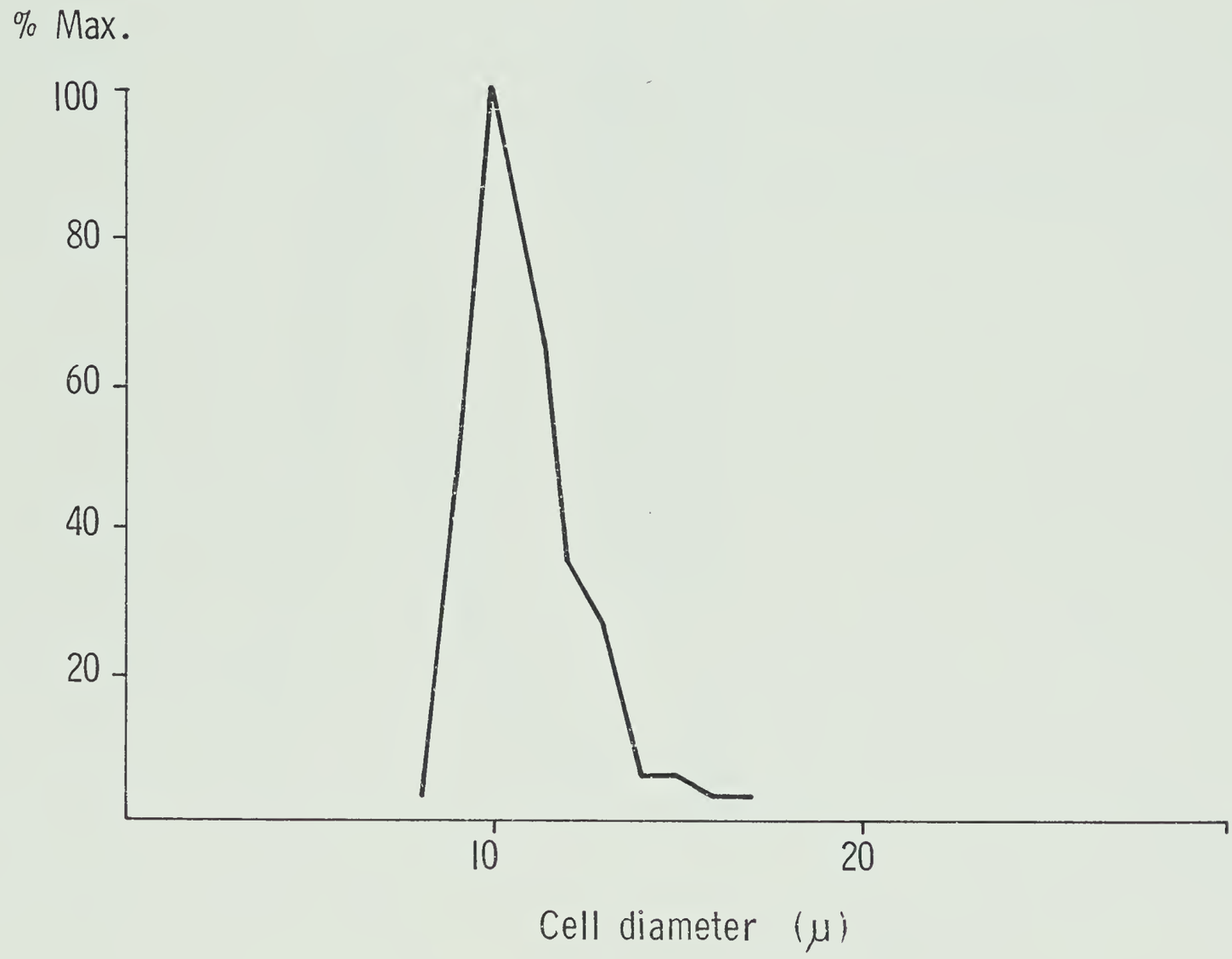

% Max.
100
80
60
40
20
10
20
Cell diameter (μ)

Figure 34 Comparison of measured and calculated sedimentation profiles
(5 day *in vivo*, 1-2 day *in vitro* tumour)

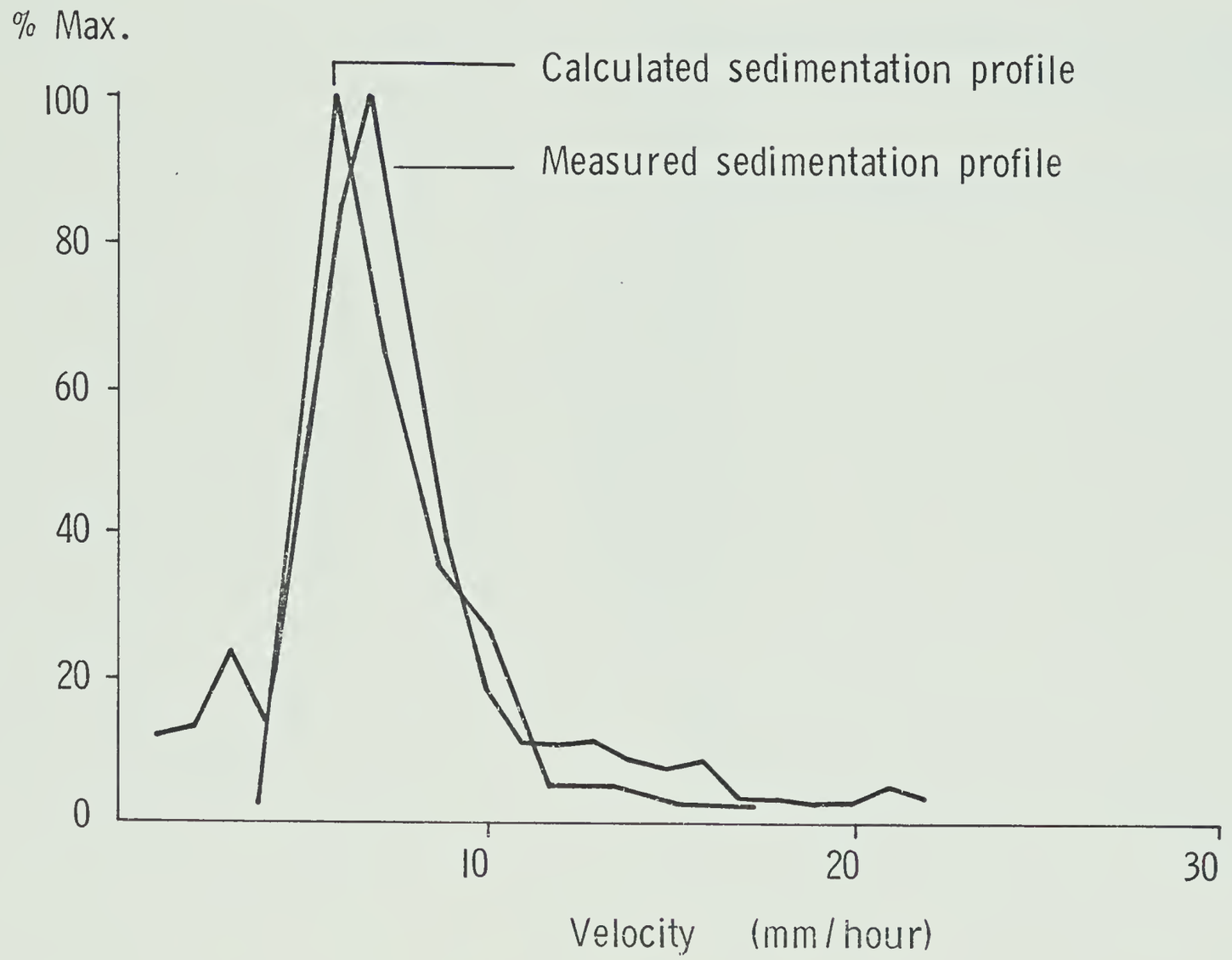

% Max.
Calculated sedimentation profile
Measured sedimentation profile
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)

Figure 35 Comparison of composite profiles for 5 day *in vivo* tumour and 5 day *in vivo*, 1-2 day *in vitro* tumour

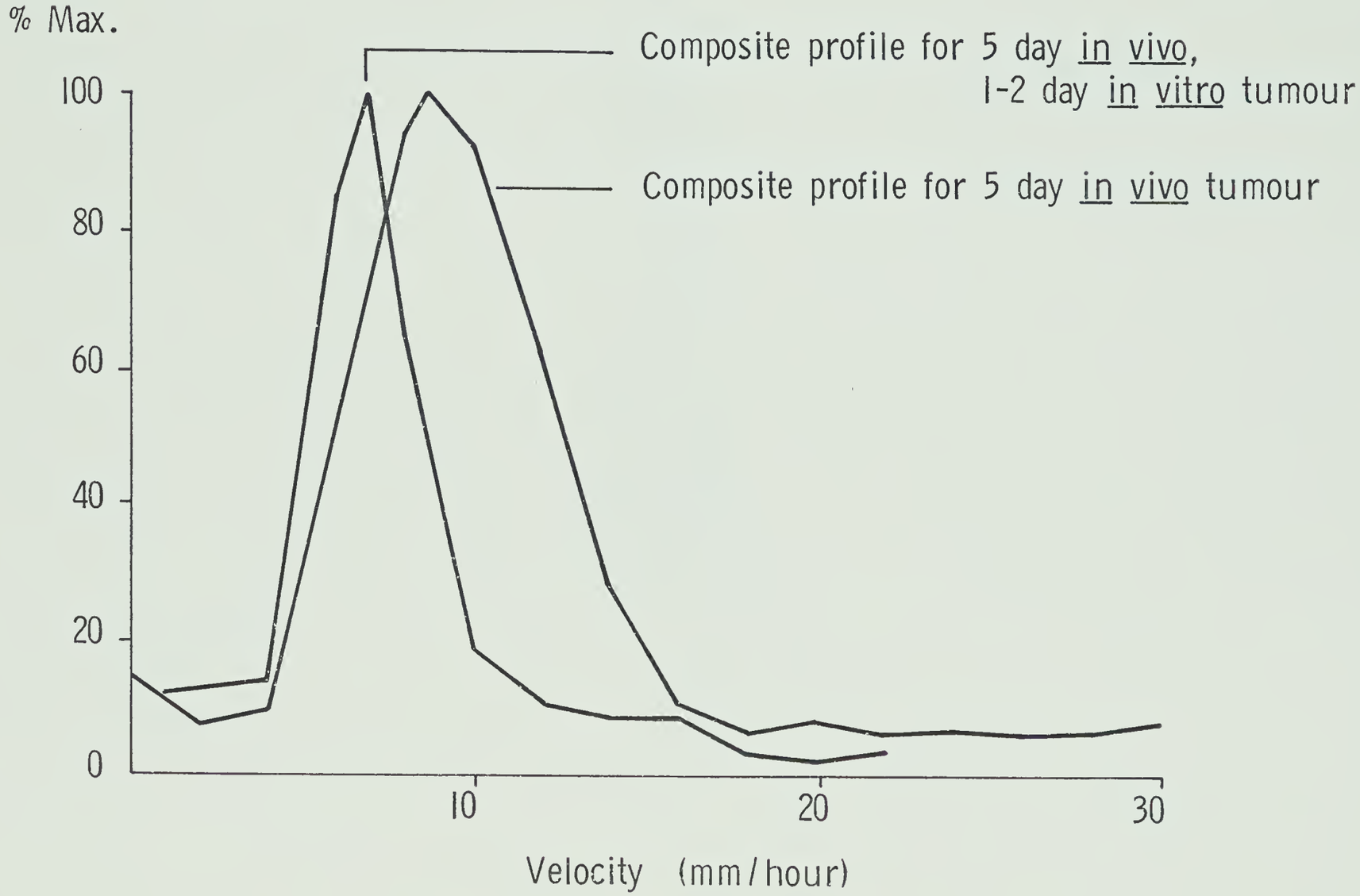
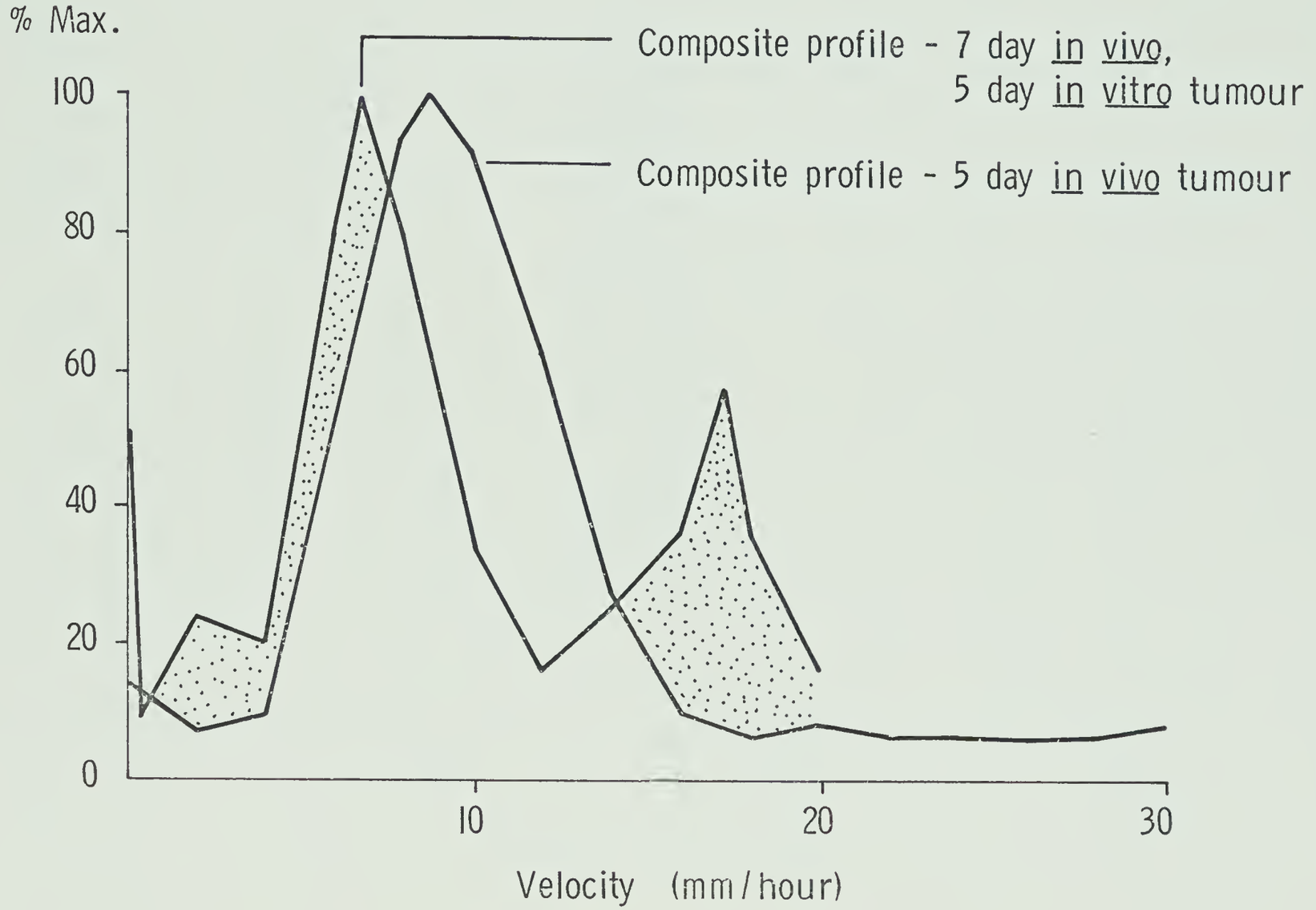

% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)
Composite profile for 5 day in vivo,
1-2 day in vitro tumour
Composite profile for 5 day in vivo tumour

Figure 36 Comparison of composite profiles for 5 day *in vivo* tumour and 7 day *in vivo*, 5 day *in vitro* tumour


% Max.
100
80
60
40
20
0
10
20
30
Velocity (mm/hour)
Composite profile - 7 day in vivo,
5 day in vitro tumour
Composite profile - 5 day in vivo tumour

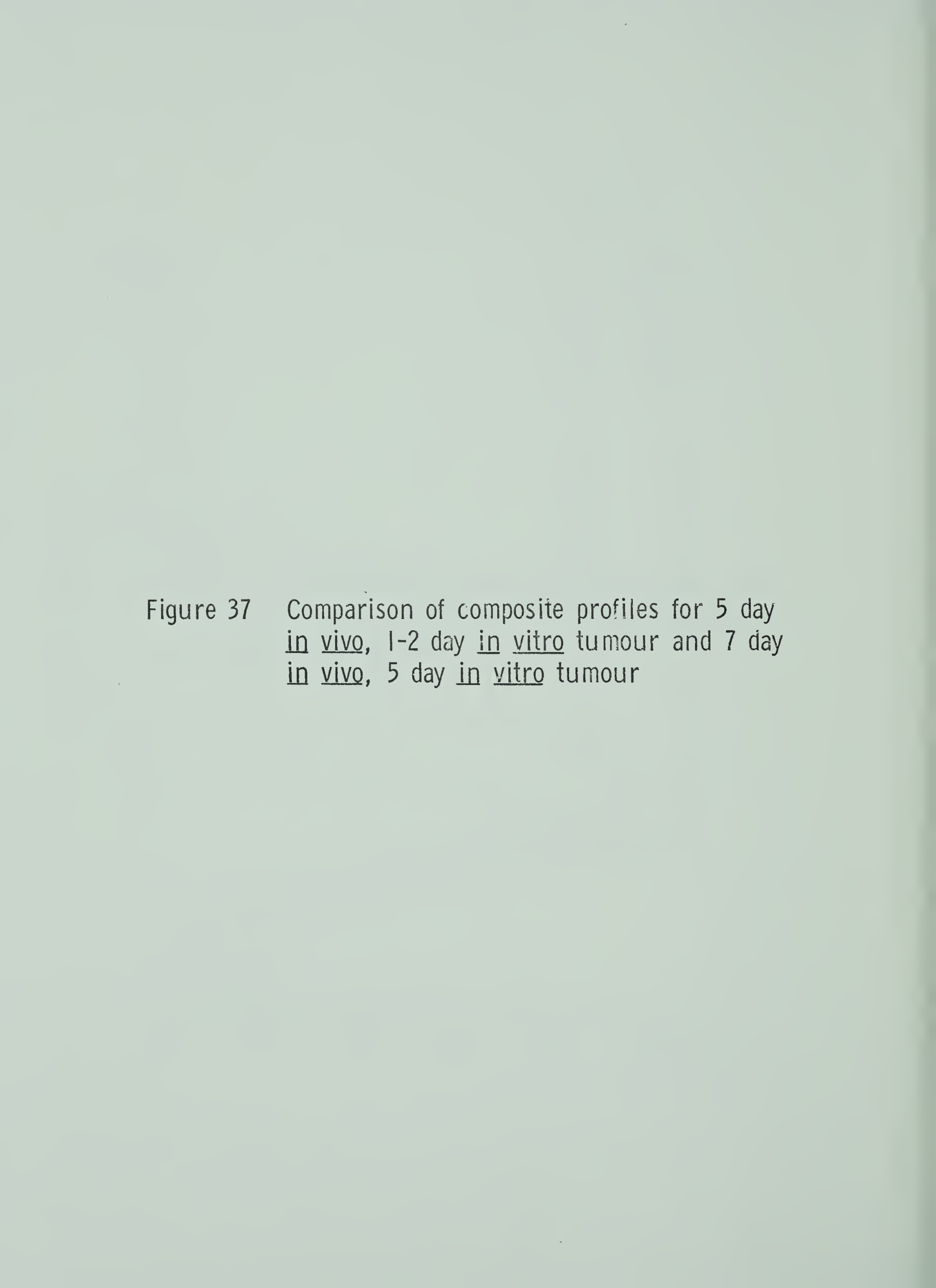
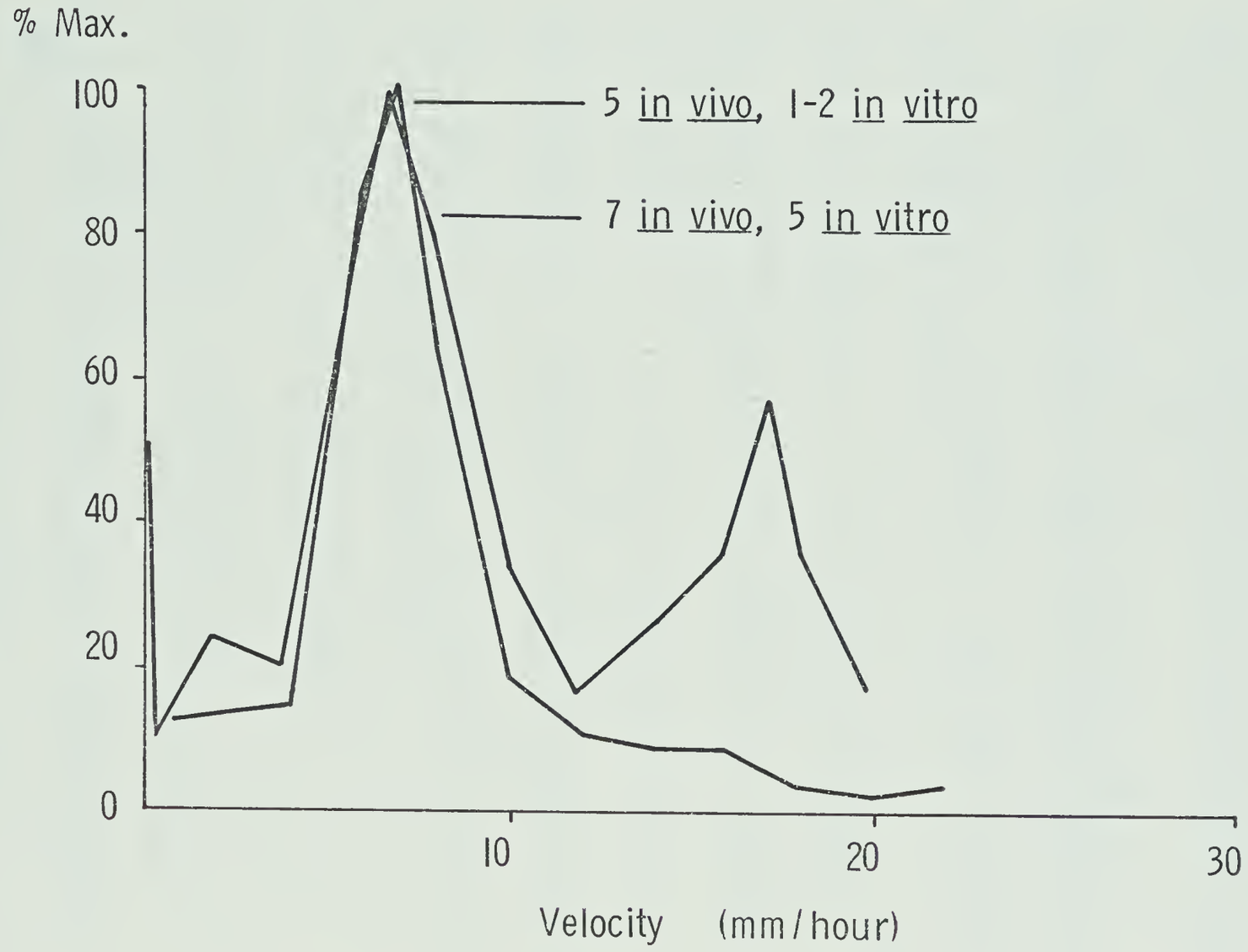


Figure 37 Comparison of composite profiles for 5 day *in vivo*, 1-2 day *in vitro* tumour and 7 day *in vivo*, 5 day *in vitro* tumour


% Max.
100
80
60
40
20
0
5 in vivo, 1-2 in vitro
7 in vivo, 5 in vitro
10
20
30
Velocity (mm/hour)

REFERENCES

ABDOU, N.I. and RICHTER, M. Cells involved in the immune response. X. The transfer of Ab-forming capacity to irradiated rabbits by Ag-reactive cells isolated from normal allogeneic rabbit bone marrow after passage through Ag-sensitised glass bead columns. J. Exp. Med. 130: 141, 1969.

ABELOFF, M.D., MANGI, R.J., PRETLOW, T.G. and MARDINEY, M.R., Jr. Isolation of human leukemic blasts from peripheral blood by density gradient centrifugation. Clin. Res. 17: 318, 1969 (Abstr.).

AGRANOFF, B.W., VALLEE, B.L. and WAUGH, D.F. Centrifugal subfractionation of polymorphonuclear leukocytes, lymphocytes and erythrocytes. Blood 9: 804, 1954.

ALEXANDER, R.F. and SPRIGGS, A.I. The differential diagnosis of tumour cells in circulating blood. J. Clin. Path. 13: 414, 1960.

ALLFREY, V. The isolation of subcellular components. In: The Cell. Vol. I, p. 193. J. Brachet and A.E. Mirsky (Eds.) New York: Academic Press, 1959.

BENNETT, W.E. and COHN, Z.A. The isolation and selected properties of blood monocytes. J. Exp. Med. 123: 145, 1966.

BLOOM, B.R. and BENNETT, B. Mechanism of a reaction *in vitro* associated with a delayed-type hypersensitivity. Science 153: 80, 1966.

BOONE, C.W., HARELL, G.S. and BOND, H.E. The resolution of mixtures of viable mammalian cells into homogeneous fractions by zonal centrifugation. J. Cell Biol. 36: 369, 1968.

BRIGGS, R. The experimental production and development of triploid frog embryos. J. Exp. Zool. 106: 237, 1947.

BRITTEN, R.J. and ROBERTS, R.B. High-resolution density gradient sedimentation analysis. Science 131: 32, 1960.

COWDREY, E.V. Cancer Cells. Philadelphia: W.B. Saunders Company, 1955.

CUDKOWICZ, G., BENNETT, M. and SHEARER, G.M. Pluripotent stem cell function of the mouse marrow 'lymphocyte'. Science 144: 866, 1964.

CUTTS, H.J. Cell Separation Methods in Haematology. New York: Academic Press, 1970.

DEDUVE, C. The scope and limitations of cell fractionation. Biochem. Soc. Symp. 23: 1, 1963. (University of Cambridge Press, Cambridge.)

DEDUVE, C., BERTHET, J. and BEAUFAY, H. Gradient centrifugation of cell particles: theory and application. Progr. Biophys. Biophys. Chem. 9: 326, 1959.

DELARUE, N.C. Clinical studies in the realm of tumour biology. Annals Roy. Col. Phys. Surg. Can. 5: 179, 1972.

DICKE, J.A., vanHOOFT, J.I.M. and vanBEKKUM, D.W. The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. II. Mouse spleen cell fractionation on a discontinuous albumin gradient. Transplantation 6: 562, 1968.

DULBECCO, R. and VOGT, M. Plaque formation and isolation of pure lines with Poliomyelitis viruses. J. Exp. Med. 99: 167, 1954.

ENGELL, H.C. Cancer cells in the circulating blood. Acta Chir. Scand. Suppl. 201: 1, 1955.

FANKHAUSER, G. The effects of changes in chromosome number on amphibian development. Quart. Rev. Biol. 20: 20, 1945.

FANKHAUSER, G. Nucleo-cytoplasmic relations in amphibian development. Int. Rev. Cytol. 1: 165, 1952.

FAWCETT, D.W., VALLEE, B.L. and SOULE, M.H. A method for concentration and segregation of malignant cells from bloody, pleural and peritoneal fluids. Science 111: 34, 1950.

FISCHER, G.A. Studies of the culture of leukemic cells *in vitro*. Ann. N.Y. Acad. Sci. 76: 673, 1958.

FULWYLER, M.J. Electronic separation of biological cells by volume. Science 150: 910, 1965.

GOODMAN, J. Bone marrow cell separation studies. Exp. Cell Res. 21: 88, 1960.

HASKILL, J.S. and MOORE, M.A.S. Two dimensional cell separation: comparison of embryonic and adult haemopoietic stem cells. Nature 226: 853, 1970.

HAUSCHKA, T.S., GRINNELL, S.T., REVESZ, L. and KLEIN, G. Quantitative studies on the multiplication of neoplastic cells *in vivo*. IV. Influence of doubled chromosome number on growth rate and final population size. J. Nat. Canc. Inst. 19: 13, 1957.

HILAL, S.K., MOSSER, D.G., LOKEN, M.K. and JOHNSON, R.W. A new technique for high-resolution density gradient separation of bone marrow cells. Ann. N.Y. Acad. Sci. 114: 661, 1964.

HIRSCHBOECK, J.S. Lecithin and the erythrocyte factor in the blood sedimentation phenomenon. Blood 2: 578, 1947.

KRAFT, N. Differentiation of the Ab-forming Cell. Ph.D. Thesis, University of Melbourne, 1972.

KUPER, S.W.A., BIGNALL, J.R. and LUCKOCK, E.D. A quantitative method for studying tumour cells in blood. Lancet 1: 852, 1961.

LEGGE, D.G. and SHORTMAN, K. The effect of pH on the volume, density and shape of erythrocytes and thymic lymphocytes. Brit. J. Haemat. 14: 323, 1968.

LEIF, R.C. and VINOGRAD, J. The distribution of buoyant cell density of human erythrocytes in Bovine Albumin solutions. PNAS 51: 520, 1969.

LEVINE, S. Magnetic techniques for *in vitro* isolation of leukocytes. Science 123: 185, 1956.

LI, J.G. and OSGOOD, E.D. A method for the rapid separation of leukocytes and nucleated erythrocytes from blood or marrow with a phytohemagglutinin from red beans *(Phaseolus vulgaris)*. Blood 4: 670, 1949.

LOEWENSTEIN, W.R. Transfer of information through cell junctions and growth control. Can. Cancer Conf. 8: 162, 1969.

McDONALD, H.R. and MILLER, R.J. Synchronisation of mouse L-cells by a velocity sedimentation technique. Biophys. J. 10: 834, 1970.

MAGE, M.G., EVANS, W.H. and PETERSON, E.A. Enrichment of antibody plaque-forming cells of spleen by sedimentation at unit gravity. Proc. Soc. Exp. Biol. Med. 127: 478, 1968.

MEL, H.C. New method of continuous free boundary electrophoresis. J. Chem. Phys. 31: 559, 1959.

MEL, H.C. Electrophoretic interaction studies by the stable-flow free-boundary method. Science 132: 1255, 1960.

MEL, H.C. Cellular fractions from stable-flow free boundary sedimentation of rat bone marrow. Blood 25: 501, 1963a.

MEL, H.C. Sedimentation properties of nucleated and non-nucleated cells in normal rat bone marrow. Nature 200: 423, 1963b.

MEL, H.C. Stable-flow free-boundary migration and fractionation of cell mixtures. I. Apparatus and hydrodynamic feedback principles. J. Theoret. Biol. 6: 159, 1964a.

MEL, H.C. Stable-flow free-boundary migration and fractionation of cell mixtures. II. Laminar flow and density gradient stability. J. Theoret. Biol. 6: 181, 1964b.

MEL, H.C. Stable-flow free-boundary (STAFLO) migation and fractionation of cell mixtures. III. Migration principles in sedimentation and electrophoresis. J. Theoret. Biol. 6: 307, 1964c.

MEL, H.C., MITCHELL, L.T. and THORELL, B. Continuous free-flow fractionation of cellular constituents in rat bone marrow. Blood 25: 63, 1965.

MILLER, R.G. and PHILLIPS, R.A. Separation of cells by velocity sedimentation. J. Cell Physiol. 73: 191, 1969.

MILLER, R.G. and PHILLIPS, R.A. Sedimentation analysis of the cells in mice required to initiate an *in vivo* immune response to sheep erythrocytes. Proc. Soc. Exp. Biol. Med. 135: 63, 1970a.

MOSIER, D.E. A requirement for two cell types for Ag-formation *in vitro*. Science 158: 1573, 1967.

OSOBA, D. Some physiological and radiobiological properties of immunologically reactive mouse spleen cells. J. Exp. Med. 132: 368, 1970.

PETERSON, E.A. and EVANS, W.H. Separation of bone marrow cells by sedimentation at Unit Gravity. Nature 214: 824, 1967.

PHILLIPS, R.A. and MILLER, R.G. Antibody-producing cells: analysis and purification by velocity sedimentation. Cell Tissue Kinet. 3: 263, 1970b.

PONDER, E. Sedimentation : Blood. In: Medical Physics. Vol. I, p. 1408. O. Glasser (Ed.) Chicago: The Year Book Publishers, Inc., 1947.

POOL, E.H. and DUNLOP, G.R. Cancer cells in the blood stream. Am. J. Cancer 21: 99, 1934.

PRETLOW, T.G. and BOONE, C.W. Resolution of viable cell mixtures using Ficoll gradient centrifugation. Fed. Proc. 27: 545, 1968a.

PRETLOW, T.G. and BOONE, C.W. Centrifugation of mammalian cells on gradients: a new rotor. Science 161: 911, 1968b.

PRETLOW, T.G. and BOONE, C.W. Separation of mammalian cells using programmed gradient sedimentation. Exp. Mol. Path. 11: 139, 1969.

PRETLOW, T.G., BOONE, C.W. and RILEY, W.B. Cell separations using programmed gradient sedimentation. Fed. Proc. 28: 684, 1969, (Abstr.).

PRETLOW, T.G. and CASSAY, I.M. Separation of mast cells in successive stages of differentiation using programmed gradient sedimentation. Am. J. Pathol. 61: 323, 1970.

PRETLOW, T.G., PICHICHERO, M.E. and HYAMS, L. Separation of lymphocytes and macrophages from suspensions of guinea pig peritonitis exudate cells using programmed gradient sedimentation. Am. J. Pathol. 63: 255, 1971.

RABINOWITZ, Y. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns including tissue culture observations. Blood 23: 811, 1964.

SCHMID, F.A. and HUTCHISON, D.J. Collateral sensitivity of resistant lines of mouse leukemias L1210 and L5178Y. Canc. Res. 32: 808, 1972.

VAN DILLA, M.A., FULWYER, M.J. and BOONE, T.U. Volume distribution and separation of normal human leukocytes. Proc. Soc. Exp. Biol. Med. 125: 367, 1967.

WEATHERALL, D.J. The red cell, physiology and pathology. In: Scientific Foundations of Surgery. C. Wells and J. Kyle (Eds.) London: William Heinemann Medical Books Ltd., 1967.

WIGZELL, H. and ANDERSSON, B. Cell separation on antigen-coated columns: Elimination of high rate antibody-forming cells and immunological memory cells. J. Exp. Med. 129: 23, 1969.

WORTON, R.G., McCULLOCH, E.A. and TILL, J.E. Physical separation of hemopoietic stem cells from cells forming colonies in culture. J. Cell Physiol. 74: 171, 1969.